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# The Morphological and Molecular Microevolution and Development of *Mimulus guttatus* (Phrymaceae) Shoot Architecture

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THE MORPHOLOGICAL AND MOLECULAR MICROEVOLUTION AND  
DEVELOPMENT OF MIMULUS GUTTATUS (PHYRMACEAE) SHOOT ARCHITECTURE

by

ROBERT L. BAKER

B.A., Reed College, 2002

A thesis submitted to the

Faculty of the Graduate School of the

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This thesis entitled:  
The Morphological and Molecular Microevolution and Development of *Mimulus guttatus*  
(Phrymaceae) Shoot Architecture  
Written by Robert Leo Baker  
Has been approved for the Department of Ecology and Evolutionary Biology

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Date\_\_\_\_\_

The final copy of this thesis has been examined by the signatories, and we  
Find that both the content and the form meet acceptable presentation standards  
Of scholarly work in the above mentioned discipline.

Baker, Robert Leo (Ph.D., Ecology and Evolutionary Biology)

The Morphological and Molecular Microevolution and Development of *Mimulus guttatus*

(Phrymaceae) Shoot Architecture

Thesis directed by Professor Pamela K. Diggle

Modern studies of evolutionary developmental (evo-devo) biology explore the molecular and developmental basis of morphological novelty, diversification, and deep homologies. Such studies primarily have focused on macroevolutionary problems by comparing developmental patterns across broad phylogenetic distances. However, natural selection and other evolutionary processes occur at the level of populations. Whether the key genes and genetic networks identified by evo-devo studies in model organisms also are responsible for morphological variation at the population level, where evolutionary processes such as selection act, is not clear. I address this problem by examining the morphological and molecular developmental basis for intraspecific variation of shoot architecture in plants from two locally adapted populations (DUN and IM) of *Mimulus guttatus* (monkeyflower) with contrasting flowering times, life histories, and branch numbers. I grew second-generation seed in growth chambers to control for maternal and environmental effects.

At the third and more distal nodes of the main axis, heterochronic processes contributed to flowering time and branch number differences between populations. Most branches, however, occurred at nodes one and two. At these nodes, branching occurred earlier and with greater frequency in DUN compared to IM plants. Plants from both populations initiate axillary meristems at the same time. At node 1, axillary meristems from both populations are vegetative; branch number at node 1 is determined by the frequency of meristem outgrowth. At node 2,



axillary meristems of DUN plants are vegetative whereas meristems of IM plants are either vegetative or floral. Differences in branch number at node 2 are caused by differences in both axillary meristem fate and the frequency of vegetative axillary meristems outgrowth.

I provide some of the first quantitative molecular genetic data from plants that associates developmental genes with intraspecific, natural variation in a functionally and evolutionarily critical aspect of morphology over the course of development. I identified four putative *M. guttatus* *MORE AXILLARY GROWTH* (*MAX*) orthologs. In *Arabidopsis* (and other model species), *MAX* genes and their orthologs negatively regulate branch outgrowth. Consistent with this function, *MgMAX1*, 2, 3, and 4 are all expressed at significantly higher levels in unbranched IM plants than highly branched DUN plants.

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## CHAPTER 1

### ABSTRACT

*Premise of the study* – Shoot architecture is a fundamentally developmental aspect of plant biology with implications for plant form, function, reproduction, and life history evolution. *Mimulus guttatus* is morphologically diverse and becoming a model for evolutionary biology. Shoot architecture, however, has never been studied from a developmental perspective in *M. guttatus*.

*Methods* – I examined the development of branches and flowers in plants from two locally adapted populations of *M. guttatus* with contrasting flowering times, life histories, and branch numbers. I planted second-generation seed in growth chambers to control for maternal and environmental effects.

*Key results* – Most branches occurred at nodes one and two of the main axis. Onset of branching occurred earlier and at a greater frequency in perennials than in annuals. In perennials, almost all flowers occurred at the fifth or more distal nodes. In annuals, most flowers occurred at the third and more distal nodes. Accessory axillary meristems and higher-order branching did not influence shoot architecture.

*Conclusions* – I found no evidence for trade-offs between flowers and branches because axillary meristem number was not limiting: a large number of meristems remained quiescent. If, however, quiescence is a component of meristem allocation strategy, then meristems may be limited despite presence of quiescent meristems. At the two basalmost nodes, branch number



was determined by mechanisms governing either meristem initiation or outgrowth, rather than flowering vs. branching. At the third and more distal nodes, heterochronic processes contributed to flowering time and branch number differences between populations.

## INTRODUCTION

As Goethe (1790) first posited, the shoot is composed of only two vegetative organ types: leaf and stem. This seemingly simple body plan has been reiterated and modified over the course of evolution, resulting in vast morphological variation among species. Much of this diversity in plant form is dictated by the location, frequency, and timing of branch outgrowth (Hallé et al., 1978; Bell, 1991; Barthélémy and Caraglio, 2007). Branch development also contributes to variation in shoot architecture within species, where this variation influences vegetative function and reproductive output.

Branching determines leaf placement and light interception, which in turn affect performance. Branching also can have a more direct impact on reproductive success. In most angiosperms, branches are the products of axillary meristems, and meristem fate (quiescent, vegetative, or floral) may determine the number of subsequent meristems available for sexual (Geber, 1990; Lortie and Aarssen, 1997, 2000b) and asexual (Watson, 1984) reproduction. Therefore, meristem fate plays a key role in determining plant fitness and life-history trade-offs (Geber, 1990; Lortie and Aarssen, 2000a). A greater number of branches may increase overall reproductive output by providing additional opportunities for sexual and vegetative reproduction (Geber, 1990; Farnsworth and Niklas, 1995; Lortie and Aarssen, 1997, 2000a). Early branch production may preclude early flowering; yet, in short or unpredictable growing seasons, plants may ensure reproduction and maximize fitness by dedicating meristems to flowers rather than

branches early in ontogeny (Cohen, 1971; Bonser and Aarssen, 1996). Thus, the developmental dynamics of axillary meristem fate, and specifically branch production, may be a central aspect of local adaptation and life history evolution (Torstensson and Telenius, 1986). Because the timing and pattern of branching affects crucial aspects of plant form and fitness, investigating the evolution of branch development is critical for a comprehensive understanding of the morphological and functional divergence of natural populations.

*Mimulus guttatus* DC (Phrymaceae) is becoming a model organism for studying evolution at the species and population levels (e.g., Willis, 1992, 1993a, b, 1999; Sweigart et al., 1999; Fishman et al., 2001, 2002; Kelly and Willis, 2002; Hall and Willis, 2005; Hall et al., 2006; Hall and Willis, 2006; Sweigart et al., 2006; Lowry et al., 2008, 2009; Lowry and Willis, 2010). The natural history, ecology, and population and evolutionary genetics of *M. guttatus* have been studied extensively for more than half a century (Vickery, 1956). Recently, detailed genetic maps, a sequenced and annotated genome, and searchable online databases of expressed sequence tags (ESTs) and gene orthologs have become available (Wu et al., 2008). Despite the combination of a rich ecological and evolutionary literature and the advent of impressive genetic resources, there are few studies of the developmental morphology of *M. guttatus* (for an exception, see Moody et al., 1999).

Although most studies of *M. guttatus* focus on floral characters, mating systems, and reproductive isolation, population-level differences in shoot branching have been documented (Hall et al., 2006), and these differences in shoot branching are associated with the evolution of divergent life histories (Dole, 1992; van Kleunen, 2007). Two particularly well-studied and locally adapted populations of *M. guttatus* from Oregon have contrasting patterns of shoot branching (Hall et al., 2006, 2010; Hall and Willis, 2006; Lowry and Willis, 2010). Plants from

the coastal dunes (DUN) are perennials, flower relatively late in the season, and are highly branched (Hall and Willis, 2006). In contrast, individuals from Iron Mountain (IM), an alpine habitat in the Cascade Mountains, have a summer drought–enforced annual life cycle, flower early, and have far fewer branches (Hall and Willis, 2006). The developmental dynamics that underlie the contrasting branching patterns remain uncharacterized in *M. guttatus*. I conducted a comparative ontogenetic study of the DUN and IM populations of *M. guttatus* to determine whether differences in branching patterns observed in the DUN and IM populations are maintained in a common, controlled environment, and how these distinct morphologies arise during ontogeny.

## MATERIALS AND METHODS

**Description of species**—*Mimulus guttatus* (Phrymaceae) is a species complex comprising a number of morphologically diverse populations across the western North America. Plants of all populations are herbaceous, with monopodial main and lateral axes, and bear simple leaves in an opposite decussate phyllotaxy (Jepson, 1960). Two serial axillary buds per leaf axil have been reported for California and Colorado populations, and this is likely a feature of the species (Moody et al., 1999). I refer to the additional meristem as an accessory axillary meristem.

*Mimulus guttatus* flowers are showy, yellow, zygomorphic, and borne singly in leaf axils. I focus on two well-studied populations, one from the coastal dunes near Florence, OR (DUN) and the second from Iron Mountain in the Cascade Mountains of Oregon (IM; e.g., Willis, 1992, 1993a, b; Sweigart et al., 1999, 2006; Willis, 1999; Fishman et al., 2001, 2002; Hall et al., 2006; Hall and Willis, 2006; Lowry et al., 2008, 2009; Lowry and Willis, 2010). The DUN and IM localities are at roughly the same latitude (DUN, 43°53'35"N; IM 44°24'35"N; Wu et al., 2010)

and experience similar day lengths throughout the year. The IM population occurs at 1463 m on a sloping hillside where plants germinate in the spring in a seasonal seep created by snowmelt. At this site, environmental conditions fluctuate widely throughout the year in a manner typical of a temperate alpine environment. Winter temperatures are below freezing with an average snowfall of >2 m per year, while summer temperatures may be above 40°C with average cumulative precipitation during the summer months (July and August) of just 46.74 mm (Hall and Willis, 2005; Hall et al., 2006). The plants escape harsh winter temperatures and summer drought by completing their life cycle in just over a month. They germinate when the snow melts and senesce as seeping groundwater from higher elevation snowmelt ceases and summer drought conditions set in (Sweigart et al., 1999). In their natural habitat, IM plants typically grow just a few centimeters tall with few or no vegetative branches and one or occasionally two flowers (Hall and Willis, 2006).

The DUN population occurs in sandy areas past the foredunes at the Oregon Sand Dunes National Recreation Area near Florence, Oregon. Temperatures are moderate year-round and vary less than 20°C over the course of the year (Hall and Willis, 2005). At the dunes site, water availability is constant due to a high water table, average annual rainfall of 1930 mm, and frequent coastal fog. DUN plants are perennials, flower from early June through October or November, and often overwinter (Hall and Willis, 2005). At the DUN site, plants have an extended vegetative growth phase, including the production of numerous branches, some of which may help to anchor the plants in their sandy substrate and also may contribute to vegetative reproduction (Dole, 1992; Hall et al., 2006, 2010; Hall and Willis, 2006; Lowry and Willis, 2010).

***Seed collection***—During the summer of 2008, I collected maternal seed families from 30

plants per population. Plants from the DUN population occasionally reproduce asexually via rooting stolons (Hall and Willis, 2005). To reduce the chance of collecting seed from multiple ramets of the same genet, I collected seeds from DUN plants no closer than 1 m apart. Plants from the IM population are smaller, occur in a smaller area, and have not been reported to reproduce asexually in their natural environment; therefore, I collected seed from IM plants no closer than 0.33 m apart.

***Plant growth***—To control for maternal effects (Roach and Wulff, 1987; Galloway, 1995; Andalo et al., 1999) and to increase seed number, I randomly chose a subset of the field-collected seed, grew this seed in growth chambers, allowed the plants to self, and collected seed once the fruit had matured. Growth chambers were set to 16-h days at 18.8°C and 8-h nights at 14.0°C following previously reported growth conditions for *Mimulus guttatus* (Vickery, 1978).

The selfed seed were sterilized according to protocols available on the *Mimulus* Community Wiki ([http://openwetware.org/wiki/Mimulus\\_Community](http://openwetware.org/wiki/Mimulus_Community)), stratified in the dark at 4°C for 72 h, and sown in a 2 : 1 mixture of Farfard #2 soil (Conrad Farfard, Miami, Florida, USA) and perlite. Four seeds from each family were sown in each cell of six-cell planting packs by placing them on the wet soil surface and covering the seeds with a dusting of sphagnum moss, followed by bottom watering.

Approximately 2 wk after sowing (ca. 1 wk after germination), seedlings were transplanted into individual pots. The seedlings were transplanted over the course of 1 wk, but all seedlings were transplanted at the same developmental stage: cotyledons expanded and the second set of leaves just visible. During transplantation, the seedling roots were washed of all soil under tap water. Seedlings were planted in a 1 : 1 perlite : vermiculite mixture in 6.985 cm square by 7.3025 cm deep pots (Anderson Die & Mfg., Portland, Oregon, USA). To help the

plants overcome transplant stress, they were liberally bottom-watered for 1 wk. One-week post-transplant, all plants were limited to 10 mL tap water 6 d per week. On the seventh day of the week, plants were fertilized with 10 mL of a dilute fertilizer (modified Hoagland's solution with nitrogen at 200 ppm). Plants were randomly assigned to positions in one of six trays. The location of plants within trays and the location of trays within the chamber were re-randomized weekly. Because the growth chamber could only accommodate 144 plants, the selfed seeds from the same maternal plants were germinated and grown twice under the same conditions; each of the two rounds of seeds sown included representatives from both populations and all families and was considered a separate block. All plants used for this study were grown in 2009.

**Data collection**—Plants were measured once per week starting 1 wk after transplantation and continuing until the plants were 7 wk old. This growth period approximates the reported lifespan of IM plants in the field (Willis, 1993a). The following characters were measured: plant height from the soil surface to plant apex (cm), number of main axis nodes (with node 1 being the cotyledonary node), number and location of flowers, number of branches at each node on the main axis, and length and number of internodes of each branch. I defined a node as occurring when I could visually identify a leaf pair. I recorded a flower as present when a floral bud was visible. Axillary products that were visible but too small to measure with digital calipers were assigned an arbitrary length of  $<0.01$  cm and were considered quiescent axillary buds that had not yet begun to grow out as branches. All measurements were taken using digital calipers (Mitutoyo Corp., Kawasaki, Japan) or a standard metric ruler.

Sample size decreased across weeks for three reasons. First, there was some plant mortality. Second, during development some plants exhibited abnormal growth with extremely short internodes and curled leaves, which made collecting many measurements without

damaging them impossible. Other plants had aberrant growth patterns such as whorled phyllotaxy. These plants were removed from the study. Third, during the first round of planting (block 1), a subset of plants was selected for destructive sampling (for additional developmental analyses; Baker and Diggle, unpublished data). Because the destructively sampled plants were chosen nonrandomly, once plants were destructively sampled from a block, no more measurements were taken from that block. Eight hundred and twenty-six observations from 151 unique DUN plants (from 17 families) and 73 unique IM plants (from 13 families) were used in the final analyses.

**Data analysis**—I examined the effect of population and developmental time (week) on branch production using mixed model repeated measures analyses (lme functions from the nlme package of R statistical software; Pinheiro and Bates, 2000; Pinheiro et al., 2011). The full model included population, time (in weeks), block, and their interactions as independent, fixed effects and individual plant nested within family as random effects. Week was treated as a continuous variable. The effect of block was only significant when I included data from week 3. Because week 3 data were missing for one block, I also analyzed the data excluding wk 3. In this analysis, the main effect of block was no longer significant. Block was therefore removed from all subsequent analyses. Although the week 3 effect of block is evident in the graphs presented, excluding week 3 data did not change the significance of any main effects or interaction effects. Therefore, week 3 data were included in the final analyses. No adjustments were made for testing multiple hypotheses using the same data (Rothman, 1990).

For each analysis, I determined whether random effects of maternal family and/or individual were important by comparing a model with individual plant nested within family (full model) to a reduced model containing only individual plant for each of the dependent variables

using likelihood ratios. When the full model was not significantly better than the reduced model, I examined fixed effects in the reduced model, otherwise fixed effects were determined using the full model (Appendix A; Crawley, 2007). Including plant as a random variable means that my models account for repeated measures of the same individual (Crawley, 2007, p. 629); the mixed effects models I used calculate degrees of freedom based on the formulae set forth by Pinheiro and Bates (2000, pp. 91–92).

After running each model, I examined the residuals qualitatively (using qqplots; Appendix B, see online Supplemental Data). Many of my variables included numerous zeros. I used either natural log (average branch length in centimeters, plant height in centimeters) or square root (average branch length as measured by number of nodes) transformations of the data to best meet the assumptions of normality inherent in the models (Pinheiro and Bates, 2000). All data from transformed variables were back-transformed for presentation. The back-transformed means and the range limits for 95% confidence intervals are presented as appendices (online Appendices S3–S8).

Because the lme function does not allow for detailed posthoc testing, particularly of interaction effects, I visualized the data graphically in Excel. Nonoverlapping 95% confidence intervals were interpreted as a conservative estimate of significant differences ( $P < 0.01$ ) between population means for any given time point (Gardner and Altman, 1986; Yoccoz, 1991; Cumming et al., 2007).

## RESULTS

***Plant height***—The average height of IM plants as measured in centimeters increased at a greater rate than that of DUN plants (Table 1-1). This difference in growth rate between the



Table 1-1. F values from the linear mixed model analyses of plant height for plants from the Dunes and Iron Mountain populations of *Mimulus guttatus*.

Source of Variation (Df)	Dependent Variable	
	Height (cm)	Number of nodes
Population (1, 28)	0.02	0.10
Week (1, 595)	679.69***	2676.05***
Population x week (1, 595)	206.49***	3.29

Notes: Degrees of freedom (Df) are 595 rather than 600 for week and for population x week because of some missing variables. \*\*\* $P < 0.0001$ , \*\* $P < 0.01$ , \* $P < 0.05$ .

populations resulted in a significant difference in mean height that was first detected at week 2 (Fig. 1-1A). During weeks 3 and 4, the growth rate of IM plants increased, resulting in a larger difference in plant height between DUN and IM plants during weeks 4–6 compared to week 2–3. Plants of both populations ceased growth in height at week 5.

**Plant height (node number)**—The number of main axis nodes increased at the same rate in both DUN plants and IM plants (Table 1-1). The number of main axis nodes was not significantly different between the two populations at any time point (Fig. 1-1B). Plants of both populations ceased node production at week 5.

**Branch number**—All branches occurred at the first four nodes of plants from both DUN and IM populations. I often observed two buds per leaf axil; however, with the exception of a single DUN individual, only one meristem per leaf axil grew out into a branch.

**Total branch number**—The number of branches borne at each of nodes 1–4 of the main

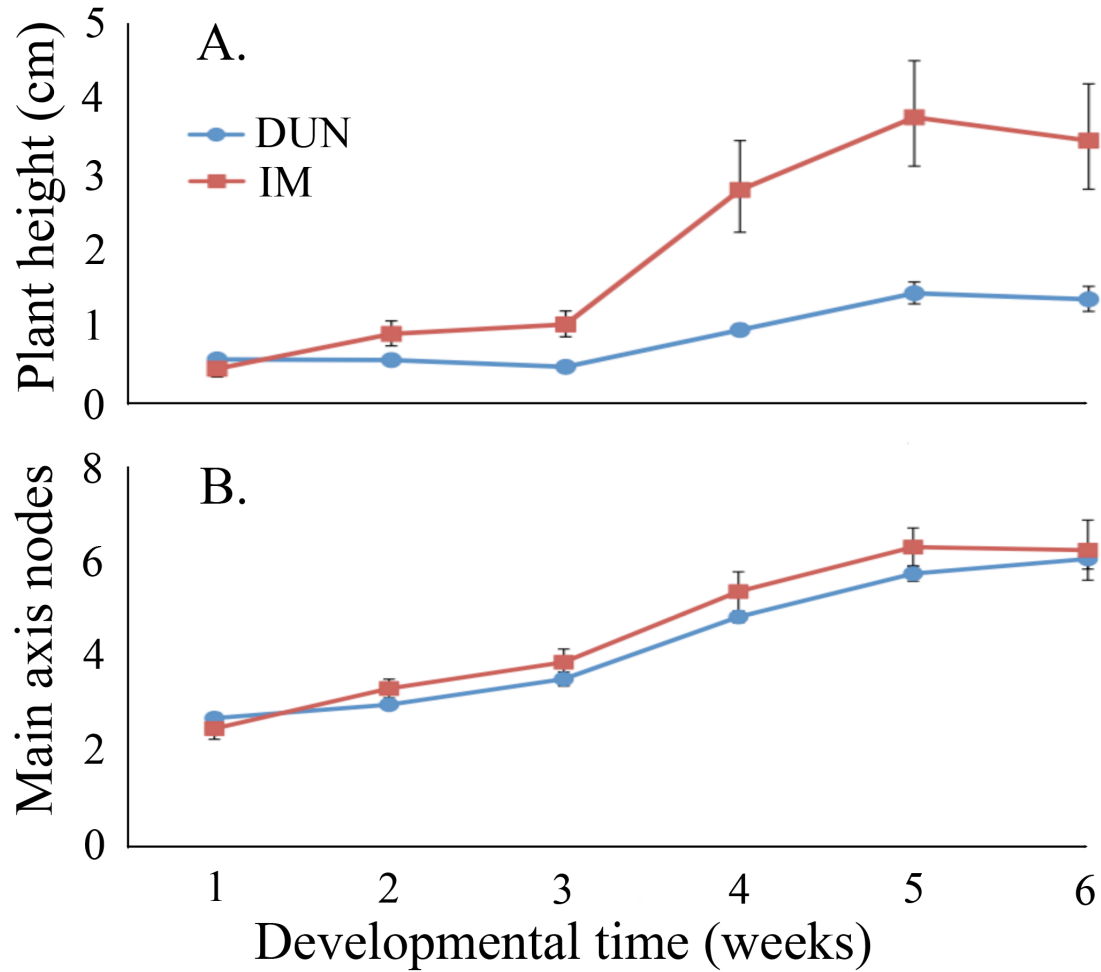


Fig. 1-1. Growth of the main axis of plants from the DUN and IM populations of *Mimulus guttatus* over the course of 6 wk demonstrates that, while (A) IM plants are taller in absolute height than DUN plants, (B) both IM and DUN plants produce nodes at the same rate. Symbols are means and 95% confidence intervals. Non-overlapping 95% confidence intervals were interpreted as a conservative estimate of significant differences between means, see Materials and Methods for details.

axis was summed to form a single variable. Plants from the DUN and IM populations differed in total branch number, and this difference changed over time (Table 1-2). The DUN plants produced branches at a greater rate than IM plants (Table 1-2; Fig. 1-2A). The difference in total branch number was first evident at week 4, and total branch number became increasingly different over time (Fig. 1-2A). Consideration of branch number at individual nodes showed that differences in branch number were not evenly distributed along the main axes. DUN plants accumulated a significantly greater number of branches over time compared to the IM plants at node 1 (Fig. 1-2B; Table 1-2), node 2 (Fig. 1-2C; Table 1-2), and node 3 (Fig. 1-2D; Table 1-2), but not node 4 (Fig. 1-2E). Branches did not occur at more distal nodes. Nodes 1–4 were present on the main axis by week 3 (Fig. 1-1), indicating that there was potential for plants of both populations to branch at all of these nodes after week 3.

*Branch number at node 1*—DUN and IM plants bear similar numbers of branches at node 1 at weeks 1–3 (Fig. 1-2B). Significant differences in branch number were first apparent at week 4 when DUN plants bore more branches per plant than IM plants. While DUN plants did not develop additional branches at node 1 after week 4, IM plants initiated additional branches between weeks 4 and 5. Nevertheless, the significant difference in branch number between the populations persisted from week 4 through the end of the observation period (Fig. 1-2B).

*Branch number at node 2*—The pattern of branch production at node 2 was similar in timing to that at node 1 (Fig. 1-2C). Branch numbers were not significantly different at weeks 1–3, but by week 4, DUN plants had initiated significantly more branches than IM plants (Fig. 1-2C). As with node 1, although IM plants continued to produce branches after week 4, the significant differences between the populations persisted.

Table 1-2. F values from the linear mixed model analyses of branch number for plants from the Dunes and Iron Mountain populations of *Mimulus guttatus*.

Source of	Dependent Variable (Df)			
Variation	Nodes 1-4	Node 1	Node 2	Node 3
Population (Pop.)	5.89* (1, 222)	0.10 (1, 222)	10.09** (1, 222)	4.79* (1, 28)
Week (wk)	1544.45*** (1, 222)	720.10*** (1, 222)	1042.25*** (1, 222)	139.90*** (1, 222)
Pop. x wk	169.23*** (1, 222)	34.89 (1, 222)	160.73 (1, 222)	38.99 (1, 222)

Notes: \*\*\* $P < 0.0001$ , \*\* $P < 0.01$ , \* $P < 0.05$ .

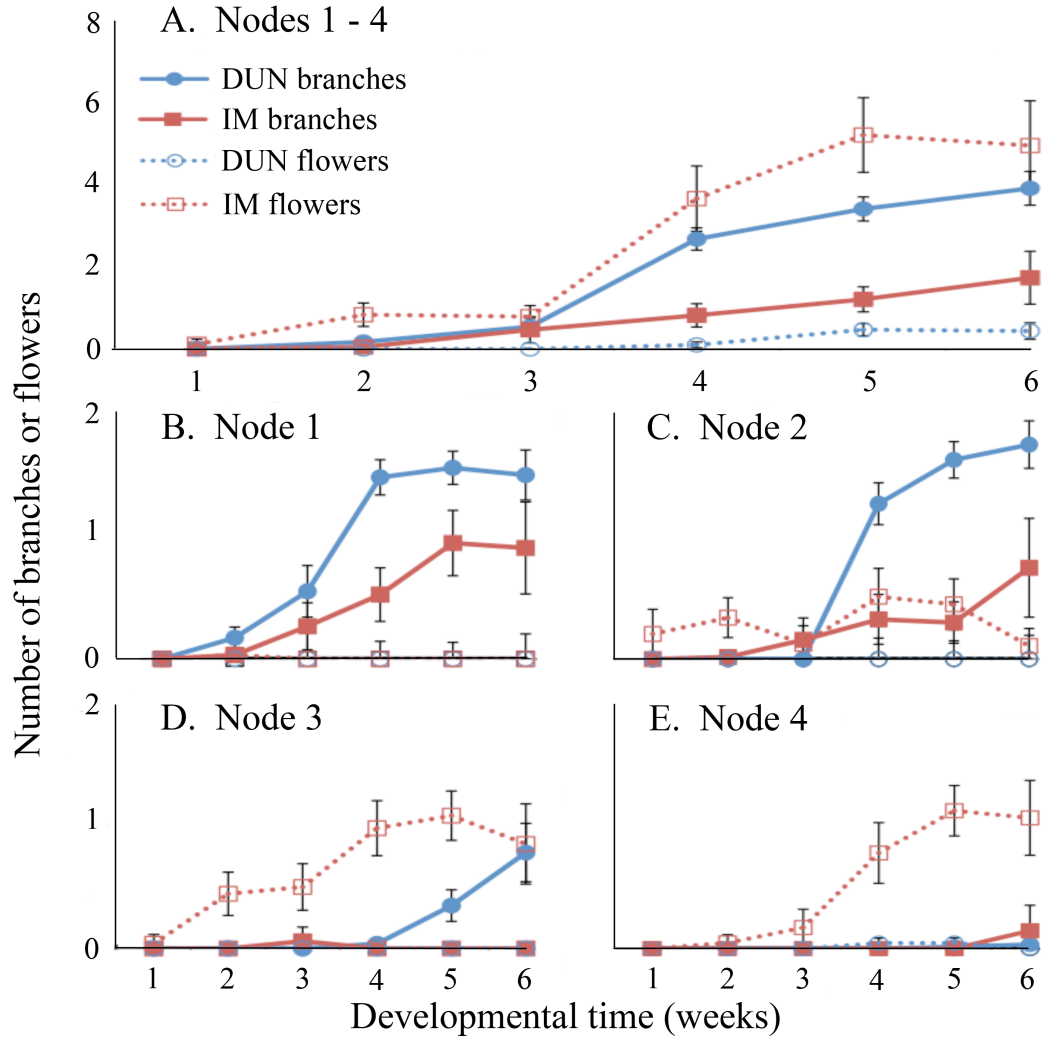


Fig. 1-2. Average total number of branches and flowers on the main axis of *Mimulus guttatus* plants from the DUN and IM populations at the basalmost four nodes, where all branches occur during the course of early ontogeny. (A) Nodes 1–4, combined. (B) Node 1. (C) Node 2. (D) Node 3. (E) Node 4. Symbols are means and 95% confidence intervals. Note that the y-axis changes from a maximum of eight in 2A (because there are eight possible leaf axils (two per node) in which to develop axillary branches or flowers) to two in (B–E) because there are only two leaf axils at each node. Non-overlapping 95% confidence intervals were interpreted as a conservative estimate of significant differences between means, see Materials and Methods for details.

*Branch number at node 3*—Differences in branch number appeared later in ontogeny at node 3 than at nodes 1 or 2 (Fig. 1-2D). Neither population bore branches until week 5, and at week 5, DUN plants had significantly more branches than IM plants, which had none. This difference persisted and increased in magnitude through week 6 when DUN plants had on average 0.78 branches, and IM plants remained unbranched at node 3.

*Branch number at node 4*—At node 4, branch number did not significantly differ from zero for both DUN and IM populations (Fig. 1-2E).

*Branch length—Total branch length*—Branch lengths were summed for each node, and these sums were again summed for nodes 1–4. Total branch length at nodes 1–4 (in cm) for DUN and IM increased at different rates during the 6 weeks of observation (Table 1-3); however, the average total branch length differed significantly between the populations only at week 4 (Fig. 1-3A). A node-by-node inspection of branch growth along the main axis suggested that the differences in total branch growth rates were caused by the combined effects of different growth rates at node 1 (Table 1-3; Fig. 1-3B), node 2 (Table 1-3; Fig. 1-3C), and node 3 (Table 1-3; Fig. 1-3D), but not node 4 (Fig. 1-3E) or subsequent nodes (data not shown). Except for at node 3, variation in IM branch length was noticeably greater than variation in DUN branch length throughout the observation period (Fig. 1-3A–E).

*Branch length at node 1*—Branch length for DUN and IM plants at node 1 remained close to 0 cm for weeks 1–3 (Fig. 1-3B). Although some plants did have branches at these times (Fig. 1-2B), average lengths close to zero reflect the large number of zero-length branches (when no branches were present) included in this calculation. Between weeks 3 and 4, branches at node 1 on DUN plants grew rapidly and by week 4, the average length of branches on DUN plants was significantly greater than that of IM plants (Fig. 1-3B). At node 1, branches on IM plants

Table 1-3.  $F$  values from the linear mixed model analysis of branch length (in cm) for plants from the Dunes and Iron Mountain populations of *Mimulus guttatus*.

Source of variation	Dependent Variable (Df)			
	Nodes 1-4	Node 1	Node 2	Node 3
Population (Pop.)	1.06 (1, 222)	0.23 (1, 222)	9.40** (1, 28)	4.76* (1, 28)
Week (wk)	1366.72*** (1, 600)	787.34*** (1, 600)	862.21*** (1, 600)	141.72*** (1, 600)
Pop. x wk	40.71*** (1, 600)	20.67*** (1, 600)	98.62*** (1, 600)	38.459*** (1, 600)

Notes: \*\*\* $P < 0.0001$ , \*\* $P < 0.01$ , \* $P < 0.05$ .

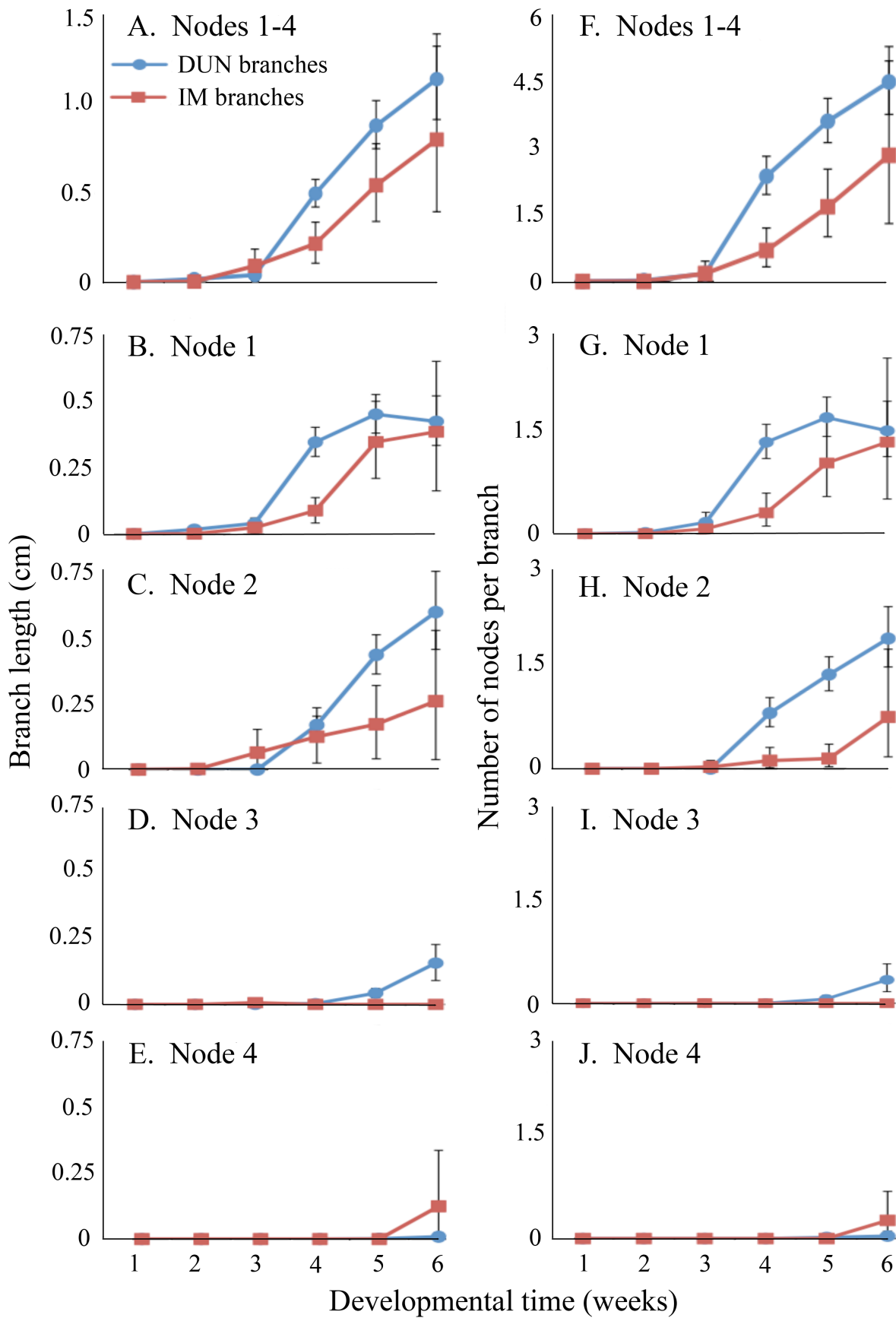




Fig. 1-3. Average total branch length (in cm) and cumulative number of nodes per branch on *Mimulus guttatus* plants from the DUN and IM populations over the course of early ontogeny. Branch length (in cm) at (A) nodes 1–4, combined. (B) Node 1. (C) Node 2. (D) Node 3. (E) Node 4. Note that the *y-axis* scale differs between (A) and (B–E). Branch lengths differed significantly only during week 4 at nodes 1 and 2 (A–C). Cumulative nodes per branch at (F) nodes 1–4, combined. (G) Node 1. (H) Node 2. (I) Node 3. (J) Node 4. Means and 95% confidence intervals were back-transformed. Note that the *y-axis* scale differs between (F) and (G–J). Non-overlapping 95% confidence intervals were interpreted as a conservative estimate of significant differences between means, see Materials and Methods for details.

followed the same pattern as DUN plants, albeit slightly later in ontogeny: their branches rapidly increased in length between weeks 4 and 5. By week 5, average IM branch length was not different from that of DUN plants (Fig. 1-3B).

*Branch length at node 2*—As with node 1, branch length at node 2 remained at or near zero for both populations during the first 3 weeks of observations (Fig. 1-3C). From weeks 3–6, the average branch length of DUN plants rapidly and steadily increased. Unlike node 1, at node 2 branches on IM plants increased in length at the same time, or slightly before, DUN plants. Node 2 branch length of DUN plants increased more rapidly than that of IM plants, resulting in significant differences in branch length between DUN and IM plants by week 5, 1 week later than at node 1 (Fig. 1-3C). As with node 1, no significant difference in branch length remained by week 6 of observation. Unlike node 1 branch length, however, this lack of statistical difference between IM and DUN plants was likely due to the large variation in branch length among individuals, especially from the IM population (Fig. 1-3C).

*Branch length at node 3*—The average branch length of both DUN and IM plants was zero at node 3 during the first 4 weeks of observation. Between week 4 and week 6, the average branch length of DUN plants increased while IM branches length remained zero (Fig. 1-3D). Note that IM branch number at node 3 also remained at zero (Fig. 1-2D). The significant differences in average branch length were first evident at week 5 and increased in magnitude through week 6.

*Branch length at node 4*—Both DUN and IM plants did not have branches through week 5 (Fig. 1-2E) and the average branch length for plants from both populations was zero through week 5. Between weeks 5 and 6, some branches of both DUN and IM plants began to elongate; however, the mean branch length of both populations was indistinguishable from zero (Fig. 1-

3E). The relatively large variance in IM branch length was due to a small number of plants that had a substantial increase in branch outgrowth.

**Nodes per branch**—*Total nodes per branch*—All branches by definition had at least one

Table 1-4. *F* values from the linear mixed model analysis of the number of nodes per branch for plants from the Dunes and Iron Mountain populations of *Mimulus guttatus*.

Source of	Dependent variable (Df)			
Variation	Nodes 1-4	Node 1	Node 2	Node 3
Population (Pop.)	0.49 (1, 222)	0.47 (1, 222)	6.96** (1, 222)	5.07* (1, 28)
Week (wk)	1320.26*** (1, 600)	710.88*** (1, 600)	895.85*** (1, 600)	149.98*** (1, 600)
Pop. x wk	34.42*** (1, 600)	8.56** (1, 600)	88.37*** (1, 600)	40.89*** (1, 600)

Notes: \*\*\* $P < 0.0001$ , \*\* $P < 0.01$ , \* $P < 0.05$ .

node. However, the back-transformed means used to generate the figures were often smaller than the raw means. The number of nodes for all branches (defined as vegetative axes >0.01 cm) at nodes 1–4 of the main axis were summed. There was no main effect of population on nodes per branch at main axis nodes 1–4 (Table 1-4). Over the course of ontogeny, however, there were significant differences in branch length (in nodes) at weeks 4 and 5, but not week 6 (Fig. 1-3F). These differences between the populations in total number of nodes per branch at weeks 4 and 5 are due to differences in growth rate (Table 1-4). Examination of branch growth rates (in terms of node production per week) at each of the first four nodes of the main axis reveals that the differences in average total number of nodes per plant can be explained by differences in rates of node production on branches at nodes 1, 2, and 3 and by population-specific differences at nodes

2 and 3 (Fig. 1-3G–I; Table 1-4). The average number of nodes per branch at node 4 on the main axis was indistinguishable from zero for both populations throughout the observation period and therefore did not contribute to differences in average number of nodes per branch (Fig. 1-3J).

*Nodes per branch at node 1 of the main axes*—The average number of nodes per branch at node 1 of the main axis did not differ significantly from zero for either population during weeks 1–3. The average number of nodes per branch can be zero even though the average branch number is greater than zero (Fig. 1-2) because the majority of plants have no branches at node 1 at week 1, and those that do have branches typically have only one node per branch. Thus, many zeros are included in the calculation of mean branch length (in nodes). Branches on DUN and IM plants both began growing by adding nodes between weeks 3 and 4. However, DUN plants had a higher rate of branch growth than IM plants, which resulted in a significantly greater average number of nodes per branch for DUN compared to IM plants by week 4 (Fig. 1-3G). Between weeks 4 and 5, branches on IM plants grew by rapidly adding nodes. Consequently, by week 5 the average number of nodes per branch on DUN and IM plants did not differ. This similarity persisted through week 6.

*Nodes per branch at node 2 of the main axis*—At node 2, the average number of nodes per branch was indistinguishable from zero for both populations during the first 3 weeks of observation because the average number of branches at node 2 was also close to zero during that time (Fig. 1-2C). As observed for node 1 branches, branches at node 2 of DUN plants began to quickly increase in length by production of new nodes between weeks 3 and 4. Branches of DUN plants continued to grow by adding nodes throughout the remainder of the study. In contrast to the pattern observed at node 1, at node 2 branches on IM plants did not begin to grow appreciably by increasing in node number until after week 5. The earlier onset of branch growth

at node 2 in DUN plants resulted in significant differences in average number of nodes per branch between DUN and IM plants at weeks 4 and 5. Between weeks 5 and 6, branches on IM plants began to increase in average node number. This growth of branches on IM plants, as well as a large increase in the variation in number of nodes per branch among IM individuals at week 6, resulted in a nonsignificant difference in average number of nodes per branch in DUN and IM plants by week 6 (Fig. 1-3H).

*Nodes per branch at node 3 of the main axis*—At node 3 on the main axis, the average number of nodes per branch was indistinguishable from zero for both populations during the first 5 weeks of observation. Between weeks 5 and 6, branches on DUN plants had increased in average node number while the average number of nodes per branch on IM plants remained zero, resulting in a significant difference in average number of nodes per branch at week 6 on node 3 of the main axis (Fig. 1-3I).

*Nodes per branch at node 4 of the main axis*—In parallel with branch number, the average number of nodes per branch at node 4 on the main axis remained indistinguishable from zero throughout the 6-week observation period for plants from both the DUN and IM populations (Fig. 1-3J).

***Flower number***—The first IM plants began flowering at week 1, and 95% of IM plants had flowered by the end of the study. Half of the flowers on IM plants occurred at the basal most four nodes of the main axis, and roughly half occurred at more distal nodes. On IM plants, 18 individuals (25%) had at least one branch that bore flowers. At week 5, 7% of IM flowers were on branches. By week 6, 22% of IM flowers were borne on branches. However, two plants accounted for 61% of all week 6 flowers that were borne on branches. In contrast, no DUN plants flowered before week 4, and the majority of DUN plants (71%) did not flower during the

observation period. In plants from the DUN population, almost all flowers occurred on the main axis at node 5 and more distal nodes (Appendix H). Branches of DUN plants did not bear flowers.

*Flowers at nodes 1–4 of the main axis*—IM plants did not bear flowers at node 1, and rarely bore flowers at node 2 (Fig. 1-2B, C; Appendix I), but did have flowers at node 3 and later nodes (Figs. 1-2D, E; Appendix H). DUN plants did not bear flowers at nodes 1–3 and only very rarely produced flowers at node 4 (Figs. 1-2D, E, Appendix I).

To further explore the relationship between branching and flowering, I accounted for all possible developmental fates of axillary meristems borne on the main axes of week 6 DUN and IM plants at nodes 1–4 (where all branches occurred; Fig. 1-4). At these first four nodes, eight meristems (four nodes  $\times$  two leaf axils per node) per plant can grow out as branches, become flowers, or remain quiescent. Although *M. guttatus* likely bears two meristems per leaf axil, I did not consider the accessory axillary meristems in this analysis because, with the exception of a single DUN plant, accessory axillary meristems never grew out into either branches or flowers.

Other populations of *M. guttatus* initiate meristems in each leaf axil close to the shoot apical meristem while leaf primordia are still quite young; therefore, I considered nodes without branches or flowers to bear quiescent axillary meristems (Moody et al., 1999). At nodes 1–4 combined, DUN and IM plants both had about the same number of quiescent meristems (Fig. 1-4). All of the nonquiescent meristems on DUN plants were branches, whereas only half of the nonquiescent meristems of IM plants were branches, the others were flowers. At node 1, DUN plants had fewer quiescent meristems than IM plants, and in both populations all nonquiescent meristems were branches. At node 2, DUN plants again had fewer quiescent meristems than IM plants and all (DUN) or most (IM) nonquiescent meristems were branches. At node 3, DUN and IM plants had roughly the same number of quiescent meristems. However, while all

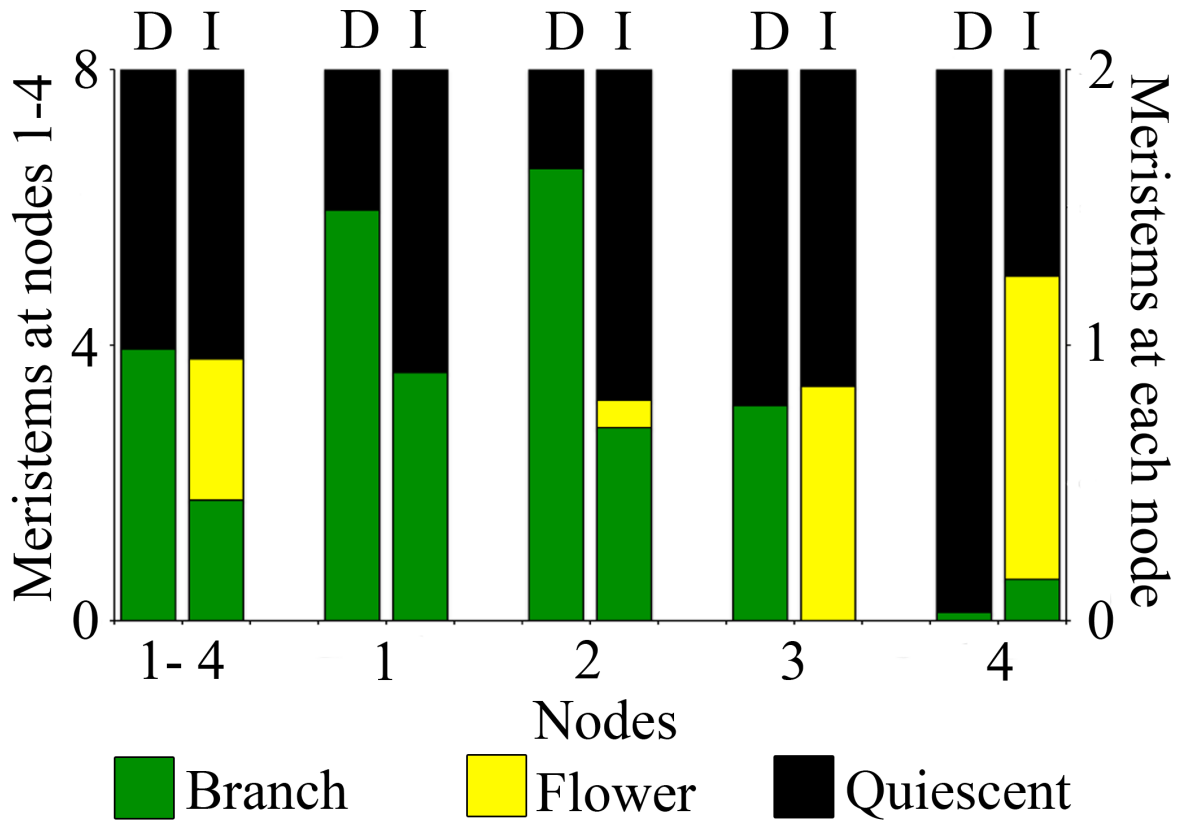


Fig. 1-4. Meristem fate at nodes 1-4 combined, and node 1, 2, 3, and 4, for plants derived from the Dunes (DUN; D) and Iron Mountain (IM; I) populations of *Mimulus guttatus* at week 6. At the first two nodes, almost all meristem products were branches, and DUN and IM plants differed in the frequency of branch outgrowth. At node 3, DUN plants produced only branches, while IM plants produced only flowers. At node 4, almost all the meristems on DUN plants remained quiescent, while IM plants produced almost exclusively flowers. Considering all four nodes, 50% of all potential meristems remained quiescent.

nonquiescent meristems of DUN plants were branches, all nonquiescent meristems of IM plants were flowers. At node 4 of DUN plants, almost all of the meristems remained quiescent, whereas at node 4, IM plants had far fewer quiescent meristems, and the majority of these nonquiescent meristems were flowers.

## DISCUSSION

The developmental fate of axillary meristems is a critical factor in the evolution of annual and perennial life histories (Torstensson and Telenius, 1986; Onishi et al., 2003). Because meristem initiation, fate, and outgrowth into branches or flowers are developmental processes, an ontogenetic approach is necessary for a complete understanding of the relationship between branching and life history. My node for node ontogenetic comparisons demonstrate that differences in branching between the two populations of *M. guttatus* result from both earlier onset as well as more frequent branching of plants derived from the DUN population compared to plants derived from the earlier flowering IM population. These patterns were predictable, repeatable, and because they are expressed in a common environment and while controlling for maternal effects, likely reflect genetic differences.

***Comparison of ontogenies***—In plants, comparative ontogenetic studies are complicated by the fact that age, size, and stage may be only loosely associated (Werner, 1975; Hubbell and Werner, 1979; Coleman et al., 1994). The number of nodes produced by a plant is a widely accepted indicator of critical ontogenetic stage and a preferred metric for comparison (Jones, 1992; Sachs, 1999). During the course of my observations, plants of both DUN and IM populations produced main axis nodes at the same rate, and so age and ontogenetic stage were equivalent under my experimental conditions (Jones, 1992). Importantly for my goal of



comparing the ontogeny of branching, plants from both DUN and IM populations produced only primary branches (branches on the main axis). Because branches are produced at nodes and DUN and IM plants have the same number of main axis nodes at every time point, from an architectural perspective, plants from each population have equal opportunities to produce meristems at each node and at each age that I observed.

***Comparison of branching characteristics***— In their natural environments, plants from the IM population rarely branch (Hall and Willis, 2006). In contrast, I observed substantial numbers of branches on plants derived from the IM population; branching clearly responds to the environment. Nevertheless, differences in branch number between plants from the DUN and IM populations persisted in the common growth chamber environment, confirming a genetic contribution to contrasting branching patterns. These results are congruent with theoretical predictions and empirical studies that compare perennials and annuals: perennials generally allocate more meristems to vegetative growth (i.e., branching) than annuals (Cohen, 1971; Bonser and Aarssen, 1996). The greater number of primary branches borne by DUN plants compared to IM plants resulted from two processes: at each main axis node DUN plants initiated branches earlier and produced branches at a greater rate (more branches per node per time interval) than IM plants. The earlier production of branches by DUN plants must be due to earlier initiation and/or earlier activation of axillary meristems at each node.

The spatial distribution of primary branches along the main axis also differed between plants from the two populations. The pattern of branching in both populations was acropetal (branches grow first from the basal most node and then sequentially at more distal nodes along the main axis) and basitonic (basal most branches were longer than more distal branches; Troll, 1959). In DUN plants, however, branches occurred at the basal most three nodes of the main axis,

whereas branching of IM plants was restricted to the first two nodes.

Initiation of two meristems per leaf axil is characteristic of *M. guttatus* (Moody et al., 1999), but accessory meristems did not contribute to branching differences in this study; I only observed multiple branches per axil on a single DUN plant. I also did not observe any higher-order branches on plants from either population. My observation period encompassed the drought-enforced life span of IM plants in their natural environment (Willis, 1993a), suggesting that accessory axillary buds and higher order branches are unlikely to contribute to branching in natural IM populations. DUN plants, however, have a longer lifespan often consisting of multiple growing seasons. Under these conditions, accessory axillary meristems and axillary meristems occurring on branches might grow out into additional branches. In their native environments, contrasting opportunities for outgrowth of accessory axillary meristems and axillary meristems occurring on branches may compound the differences I observed in primary branch number between the populations during my study.

In addition to branch number, patterns of branch elongation also differed between DUN and IM plants. In contrast to the main axis of DUN and IM plants, which produced nodes at the same rate but differed in internode elongation (Fig. 1-1), both the number of nodes per branch and length in cm were greater for DUN plants during early ontogeny. By weeks 5 and 6, the significant difference in branch length at nodes 1 and 2 (respectively) disappeared as variation in IM branch length increased dramatically compared to that of DUN plants (Fig. 1-3B, C). The contrasting variability of branch length between DUN and IM plants may be related to branching patterns expressed in their natural environments. Because DUN plants are often branched (Hall et al., 2006), the rate of branch growth (and hence length) may be under stabilizing or directional selection, which could result in relatively low levels of branch length variance (Delesalle and

Mazer, 1995). In contrast, IM plants do not normally branch in their native environment; therefore, branch length, or the rate of branch elongation, cannot be under direct selection. An association between relaxed selection and increased variance has been demonstrated using computer models (Fernandez and Caballero, 2001) and empirically (Mazer and Wolfe, 1992; Bergstrom and Reimchen, 2003; Moczek, 2010).

***Branching and flowering dynamics***—Early branching may limit early flowering and vice versa (Cohen, 1971; Geber, 1990; Bonser and Aarssen, 1996). IM plants produced more flowers, and the later-flowering perennial DUN plants produced more branches. This pattern is consistent with a direct trade-off between branching and flowering in IM and DUN plants. Indeed, when comparing total allocation of all meristems at nodes 1–4, IM plants allocated more meristems to flowers than DUN plants, and this appeared to occur at the expense of branching (Figs. 1-2A, 1-4). These differences in meristem allocation to branches vs. flowers may be related to the contrasting life histories of DUN and IM plants. Using phylogenetically independent contrasts of twenty pairs of congeneric species, Bonser and Aarssen (2006) find that at the whole plant level perennials dedicate a larger proportion of meristems to vegetative growth than annuals.

The relationship between flowers and branches in the DUN and IM populations of *M. guttatus* differs among nodes, and this relationship is complicated by the presence of quiescent meristems. According to Moody et al. (1999), axillary meristems are initiated early in the development of each node of *M. guttatus*, and thus meristems were likely present but quiescent at nodes bearing neither flowers nor branches. At nodes 1 and 2, most meristems of both DUN and IM plants have one of two developmental fates: grow out as branches, or remain quiescent (Fig. 1-4). Differences in branch number between the populations at these nodes are due to

differences in the relative numbers of quiescent vs. vegetative meristems rather than a trade-off between vegetative vs. floral fate. Only at node 3 do the populations differ in vegetative vs. floral fates of meristems: all active meristems of DUN plants develop into branches while in IM plants they all produce flowers (Fig. 1-4). Thus, a trade-off between flower and branch development might occur at this node. Yet, the large number of quiescent axillary meristems potentially available for either vegetative or floral development in both the DUN and IM plants indicates that at the morphological level allocation to branching vs. flowering is not limited by meristem number (Thiele et al., 2009). From this perspective, I find no evidence for trade-offs between flowers and branches (Watson, 1984; Geber, 1990).

Alternatively, if quiescence is viewed as part of a meristem allocation strategy, then the number of meristems available for branching or flowering may be limited despite (or because of) the presence of quiescent axillary meristems. Numerous quiescent meristems are observed across a wide range of plant taxa and are thought to act as adaptive reserves for compensatory growth in the face of herbivory or other trauma (Aarssen, 1995; Nilsson et al., 1996; Benson et al., 2004; Dalglish and Hartnett, 2006). If *M. guttatus* plants maintain a pool of quiescent meristems that are not available for immediate development, then trade-offs between branching and flowering, or among all three meristem fates, could still occur.

***Shoot architecture and life history evolution***—The evolution of diminutive size and early flowering of some annuals has been attributed to heterochronic shifts (sensu Gould, 1977) in the onset of reproduction (phase change) relative to vegetative ontogeny (Guerrant, 1988; Wiltshire et al., 1994; Diggle, 1999; Li and Johnston, 2000; Box and Glover, 2010). Earlier onset of reproduction within the context of an otherwise unchanged vegetative ontogeny (progenesis) results in plants that flower at a smaller size or “juvenile” morphology, while later reproduction

(hypermorphosis), results in greater vegetative proliferation and a morphology “beyond” (concepts in Alberch et al., 1979; extension to plants reviewed in Diggle, 1999).

A thorough analysis of heterochrony in the DUN and IM populations of *M. guttatus* is complicated by the fact that their evolutionary relationships remain unclear. Therefore, I cannot pose a specific hypothesis for the direction of possible heterochronic changes (i.e., progenesis or hypermorphosis). Nevertheless, decoupling vegetative ontogeny and the onset of reproduction may underlie the evolution of different flowering times in DUN and IM populations. The difference in flowering time corresponds to a shift in location (node number 3 vs. 5) of flowers along the main axis of plants from the two populations, while vegetative ontogeny, as assessed by main axis node production, remains identical. Such a change in onset of reproduction within the context of otherwise unchanged vegetative ontogeny suggests a process of progenesis or hypermorphosis (Fig. 1-5; Alberch et al., 1979; Jones, 1992). An alternative explanation for the differences in location of flowers, and consequently branches, is pre- or post- displacement caused by the deletion or addition of branch-bearing vegetative nodes on the main axis (Alberch et al., 1979; Alberch, 1985; Jones, 1992). DUN and IM plants lack additional morphological landmarks (such as tendrils) for unambiguously identifying equivalent nodes. Therefore, I cannot distinguish among the heterochronic processes that might underlie evolutionary developmental changes in *M. guttatus* flowering and branching. Heterochronic shifts that alter floral vs. vegetative meristem fate may be responsible for differences in branch number at node 3 and more distal nodes (Fig. 5). However, at nodes 1 and 2, where the majority of branches in both populations occur, the difference in meristem fate between populations primarily involves frequency of axillary meristem outgrowth into branches, not floral vs. vegetative fate.

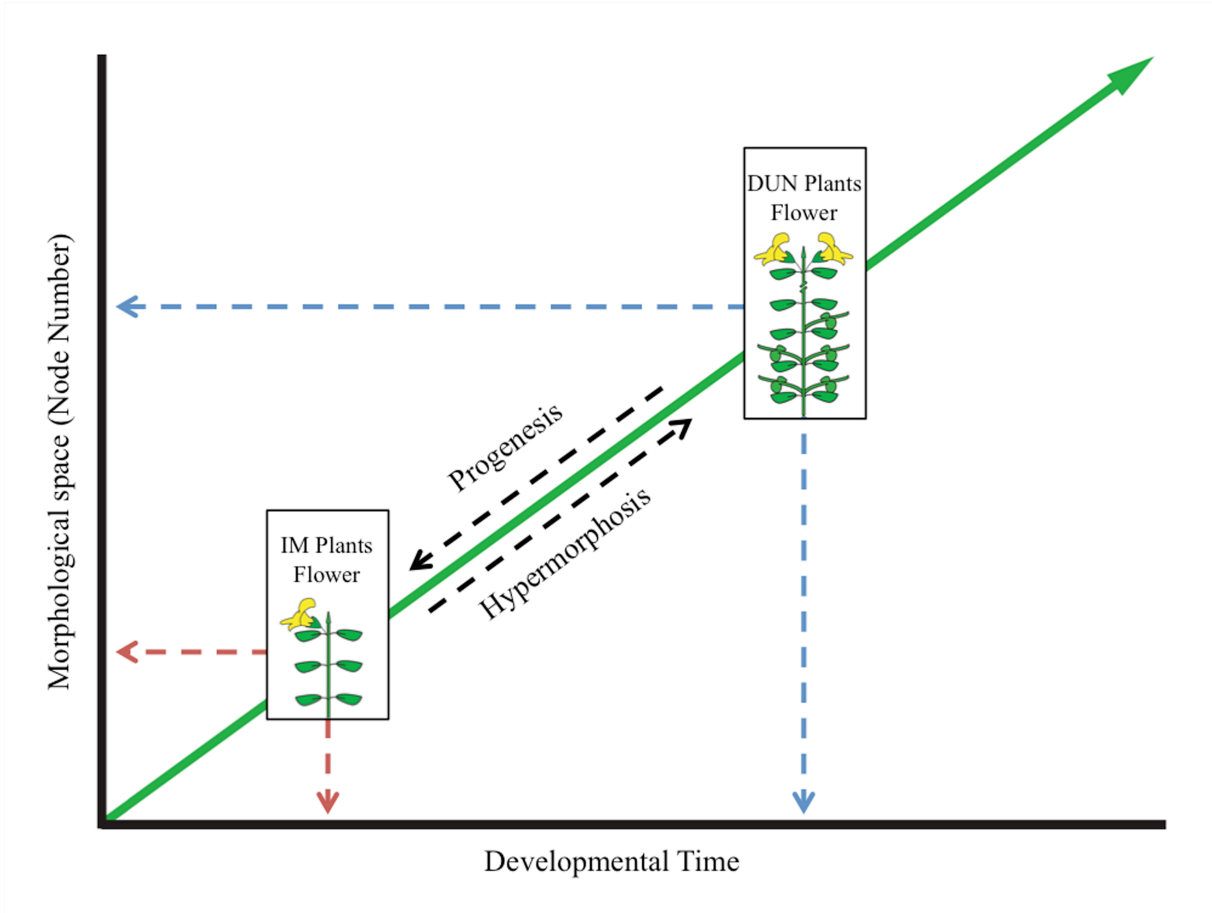


Fig. 1-5. Model of heterochronic shifts in the onset of reproduction that may explain some aspects of differences in shoot architecture between Iron Mountain (IM) and Dunes (DUN) populations of *Mimulus guttatus* (after Alberch et al., 1979). Plants from both populations go through development and explore morphological space (in terms of node number on the main axis; green arrow). IM plants flower earlier with respect to ontogenetic trajectory than DUN plants. The result is that they have a different vegetative morphology (fewer nodes) at the onset of flowering compared to DUN plants, which continue to develop vegetatively and produce additional nodes before flowering. If a DUN-like morphology were ancestral, then IM plants may have been derived via progenesis. If an IM-like morphology is a good approximation of the ancestral state, then DUN plants may have been derived via hypermorphosis.

**Conclusions**—In their native habitats, DUN plants are described as highly branched, while IM plants rarely branch. But these observations are complicated by differences in environmental conditions and life span. Using a common garden study that controls for maternal effects, I show that there are genetically determined differences in ontogenetic patterns of both branching and flowering that are clearly separable from differences in environment and life span. Differences in branch number first occur at week 4, well before IM plants typically senesce. These differences are primarily caused by earlier initiation and/or earlier outgrowth of axillary meristems as branches at the first two nodes of the main axis. At the third and more distal nodes, the onset of reproduction also affects branching frequency. At these nodes, axillary meristems are allocated to floral, vegetative, or quiescent fates. Histological studies are needed to discriminate between possible differences in meristem initiation vs. meristem outgrowth. Furthermore, these studies may reveal whether there are differences in meristem number at the population level, and if initiated but dormant, whether axillary meristems are already committed to vegetative or floral fates (e.g., Meloche and Diggle, 2001).

## CHAPTER 2

### ABSTRACT

The shoot architecture is a crucial aspect of plant function, morphological diversification, and life history evolution. The genetic controls of the expression of shoot architecture, including branch outgrowth, are well characterized in model organisms where their function is highly conserved. Yet, the role of these genes in the evolution of morphological diversity has not been explored. I identify meristem outgrowth as the primary driver of branching in two locally adapted populations of *Mimulus guttatus* with divergent branch number and life histories. In *M. guttatus*, *MORE AXILLARY GROWTH (MAX)* gene expression strongly correlates with natural variation in branch outgrowth, implying a role for this pathway in the evolutionary diversification of shoot architecture.

### INTRODUCTION

Shoot branching is a fundamental variable underlying shoot architecture (Sussex and Kerk, 2001) and the evolution of morphological diversity among plants (Barthelemy and Caraglio, 2007; Bell, 2008) from the first unbranched sporophytes to intricate patterns of axillary branching in angiosperms. Shoot architecture is also variable within species, where it influences leaf placement and light interception (King, 1998; Bell, 2008), contributes to performance (Niinemets et al., 2004), and affects fitness (Lortie and Aarssen, 2000). Because both vegetative branches and flowers develop from axillary meristems, vegetative branching may preclude



flowering, and trade-offs between flowering and branching can play a critical role in the evolution of life-history strategies (Geber, 1990; Bonser and Aarssen, 2006). Variation in the development of branches is subject to natural selection, an important component of adaptation to novel environments (Bonser and Aarssen, 1996) and is central to artificial selection leading to domestication in cereal crops (Doebley et al., 1997; Gepts and Papa, 2001). Understanding the developmental genetics of intraspecific variation in branching is critical for understanding plant evolution and may provide important insights for further crop improvement. Yet, shoot branching has not been studied from a molecular genetic perspective in natural populations, which harbor developmental variation and are where evolutionary processes such as selection and drift act

*Mimulus guttatus* is a wide-spread and morphologically diverse species that occurs throughout western North America, has a fully sequenced genome, and has been studied extensively by ecologists and evolutionary biologists (Wu et al., 2008). Two locally adapted populations of *M. guttatus* have well-characterized and contrasting life histories and patterns of branch development and present an excellent opportunity for studying natural variation in the developmental genetics of shoot branching. In their native environment, perennial plants from the coastal dunes of Oregon (DUN population) branch early and frequently. In contrast, annual alpine plants from Iron Mountain (IM population) in the Oregon Cascades rarely branch (Hall and Willis, 2005; Hall et al., 2006). These contrasting branching patterns persist when plants are grown in a common environment and maternal effects are minimized. Differences in branch number are specific to the two basal most nodes of the main axis and are expressed early during ontogeny; they are therefore not simply due to differences in lifespan (Baker and Diggle, 2011).

Here, I first demonstrate that population-level differences in DUN and IM branching patterns are primarily caused by differences in the frequency of axillary meristem outgrowth as opposed to differences in axillary meristem initiation. Second, to further understand the developmental basis of divergent branching patterns, I also examine expression of candidate genes underlying branch outgrowth in plants from the DUN and IM populations. In the model species *Arabidopsis thaliana* *MORE AXILLARY GROWTH* (*MAX*) genes function to inhibit branch outgrowth. In *A. thaliana*, there are four single copy genes in the *MAX* pathway: *AtMAX1* is a cytochrome p450 (Booker et al., 2005), *AtMAX2* is an F-box gene (Stirnberg et al., 2007), and *AtMAX3* and *AtMAX4* are carotenoid cleavage dioxygenases (Sorefan et al., 2003; Booker et al., 2004). The protein products of *AtMAX1*, 3 and 4 function in roots to convert a  $\beta$ -Carotene precursor into strigolactone, an upwardly mobile hormone (Schwartz et al., 2004). Wild-type root stocks grafted to mutant shoots are sufficient to restore the wild-type branching pattern. However, reciprocal grafts demonstrate that wildtype *AtMAX1*, 3, and 4 in shoots are also sufficient to restore a wild-type phenotype (reviewed in Beveridge and Kyoizuka, 2010). Mutant phenotypes are observed only when *AtMAX1*, 3, or 4 are knocked out in both shoots and roots (Turnbull et al., 2002; Sorefan et al., 2003; Booker et al., 2005). *AtMAX2* acts specifically in shoots at individual nodes and forms an SCF-complex with ubiquitin to negatively regulate shoot branching (Stirnberg et al., 2007) via modulating expression of *AtPIN1* (*PIN-FORMED1*), an the auxin efflux transporter (Bennett et al., 2006).

In *A. thaliana*, inactivation of any one of the four *MAX* genes results in a substantial increase in branch outgrowth compared to wildtype plants (Stirnberg et al., 2002; Booker et al., 2004; Bainbridge et al., 2005). *MAX* orthologs have been characterized in other model taxa including *Pisum*, *Petunia*, *Oryza*, and *Solanum* as well as the non-model organism *Actinidia*

*chinensis* (kiwifruit), where they all function to negatively regulate outgrowth of axillary meristems (Sorefan et al., 2003; Snowden et al., 2005; Johnson et al., 2006; Zou et al., 2006; Ledger et al., 2010; Vogel et al., 2010; Waldie et al., 2010).

The broad conservation of *MAX* genes across all flowering plants studied thus far indicates a crucial role for the *MAX* pathway in the development of shoot architecture. However, these data come primarily from laboratory mutants and transgenic analyses of model organisms, and do not address the potential role of *MAX* genes in regulating natural variation. Quantitative Trait Loci (QTL) and Linkage Disequilibrium (LD) studies indicate that *AtMAX2* and *3* are associated with differences in branching patterns among *A. thaliana* accessions (Ehrenreich et al., 2007). Thus, although the role of *MAX* genes in natural populations has never been assessed, the *MAX* pathway may be able to produce the spectrum of branching phenotypes observed within and among populations. I ask whether *MAX* expression correlates with contrasting patterns of branch outgrowth between two populations of *M. guttatus*.

## MATERIALS AND METHODS

***Description of Species*** — *Mimulus guttatus* (Phrymaceae) is a widespread species distributed across much of western North America (plants.usda.gov). Previous studies report multiple serial axillary meristems (AXMs) per leaf axil (Moody et al., 1999). The distal, primary AXM may develop into a branch or a single yellow flower while the proximal, secondary AXM remains quiescent unless products of the primary AXM are damaged (Moody et al., 1999). *M. guttatus* is characterized by an acropetal, basitonic branching pattern with most branches occurring at the two basal most nodes (Baker and Diggle, 2011).

I focus on two well-studied, locally-adapted populations (Hall and Willis, 2006) from similar latitudes that differ in number and frequency of branches and life history (Willis, 1993a, b; Sweigart et al., 1999; Willis, 1999; Fishman et al., 2002; Hall and Willis, 2005; Hall et al., 2006; Lowry et al., 2008; Lowry et al., 2009; Lowry and Willis, 2010; Baker and Diggle, 2011). Plants from the DUN population are located at sea level in the Oregon Dunes National Recreation Area. DUN plants are perennials with numerous branches that flower relatively late (Hall and Willis, 2006; Baker and Diggle, 2011). In contrast, plants from the Iron Mountain (IM) population are short-lived, alpine (occurring at 1463m) annuals. IM plants rarely branch vegetatively; instead they flower early, producing one or occasionally two flowers on a single main axis (Hall and Willis, 2006). One or two months after snowmelt, the soil dries and IM plants senesce (Hall and Willis, 2005; Hall et al., 2006).

***Seed Collection and Plant Growth*** — Seed was collected, grown, and transplanted as previously described (Baker and Diggle, 2011). I defined germination, which roughly corresponds to wk -1, as occurring when cotyledons were first visible above the soil surface. I timed all tissue collection to occur at the same developmental stages as data collection in (2011). Tissue was collected at germination, transplantation (roughly wk 0), and weekly thereafter through wk 4.

***Microscopy*** — Tissue was collected from the first two nodes of a minimum of ten plants per population per time point. “Node 1” is the cotyledonary node. All tissue was prepared as in (1999). AXMs were recorded as present when a multi-cellular dome-like structure (Scanning Electron Micrographs; SEM) that was densely staining (Light Micrographs; LM) was observed in a leaf axil. AXMs were considered vegetative buds once the first leaf pair (prophylls) had initiated or floral when the meristem began broadening and directly initiated 5 sepals without

prophylls or other vegetative appendages. Vegetative axillary buds were considered vegetative branches once they had initiated multiple leaf pairs and internode expansion had begun.

**Gene Expression Analyses** – *A. thaliana* *MORE AXILLARY GROWTH (MAX)1*, 2, 3, and 4 were used as query sequences in tBLASTx searches of the *M. guttatus* genome (DoE Joint Genome Institute, annotation v1.1, assembly v1.0; Altschul et al., 1997). Matches with E values  $< 1 \times 10^{-50}$  were considered putative *M. guttatus* MAX orthologs. Reverse transcription quantitative PCR was performed according to standard methods (Vandesompele et al., 2002; Scoville et al., 2011; detailed in appendix J) on shoot (node 1) and root tissue. Comparisons within a given tissue type and time point do not require adjustment for inter-plate variability because they were all run on a single plate. Non-overlapping standard errors were considered evidence of significant differences at the  $< 0.05$  level (Cumming et al., 2007).

## RESULTS

***At Node 1 meristem outgrowth explains differences in branch number*** — In all DUN and IM plants examined, two serial AXillary Meristems (AXMs) were observed at each leaf axil at the first node of the main axis. For plants of both populations, the primary AXM was always vegetative. The secondary AXM was also vegetative, initiated after and proximal to the primary AXM, and never developed more than one set of leaves (prophylls).

At germination neither DUN nor IM plants bear AXMs at node 1 (Fig. 2-1 B-E). Plants from both DUN and IM populations had initiated a single primary AXM in each leaf axil at wk 0.

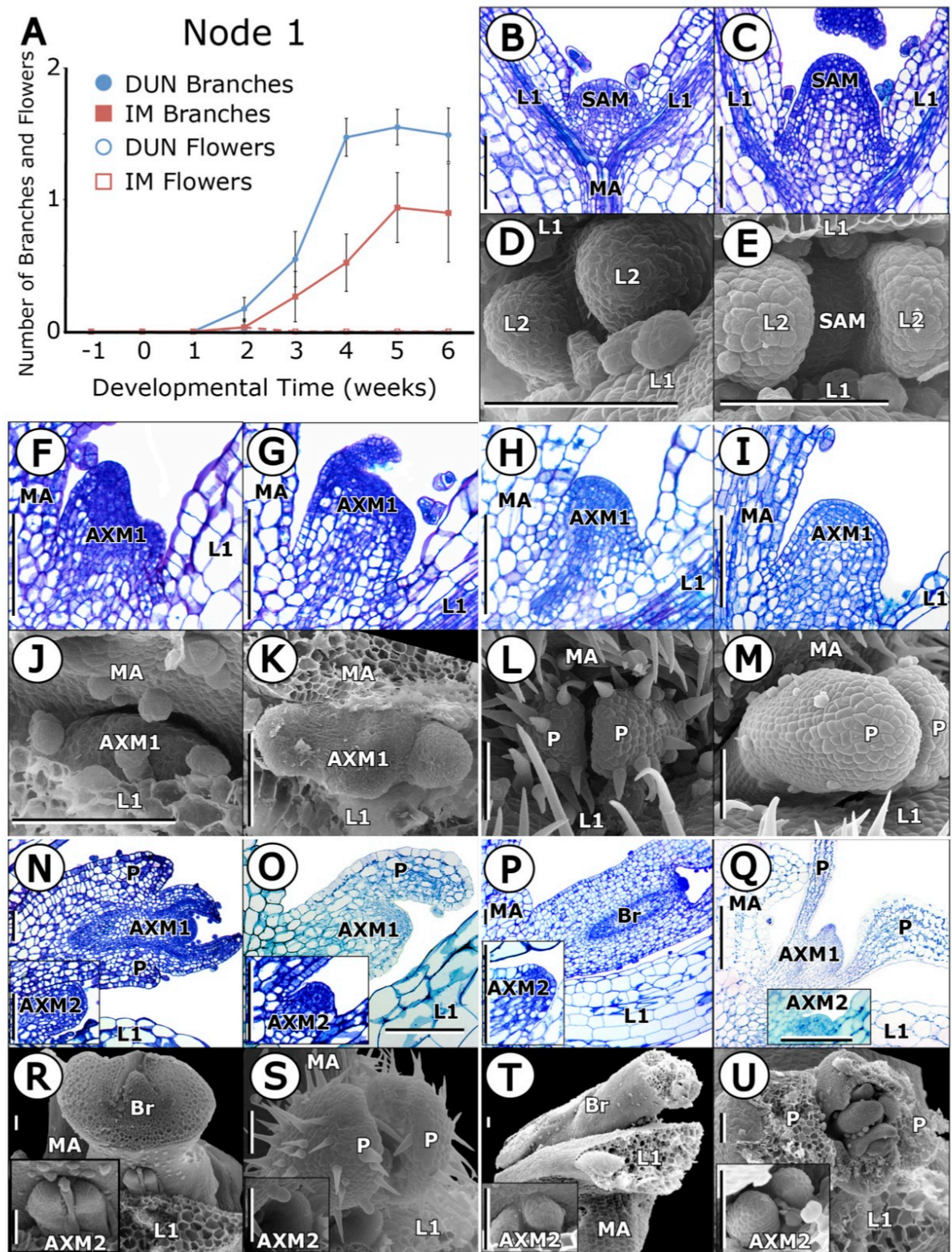


Fig. 2-1. Axillary meristem development at node 1 on the main axis of plants from the Dunes and Iron Mountain populations of *Mimulus guttatus*. (A) Branch number as a function of developmental time for plants of the Dunes (DUN) and Iron Mountain (IM) populations of *M. guttatus*. (A) By week 4 DUN plants bear significantly more branches than IM plants (adapted from Baker and Diggle, 2011). (B-C) longitudinal section of SAM at germination (wk -1), note absence of AXM. (B) DUN node 1. (C) IM node 1. (D-E) SEM of SAM at germination (wk -1) following initiation of second node. (D) DUN (E). IM. (F-G) longitudinal section of node 1 at wk 0. (F) DUN. (G) IM. (H-I) longitudinal section through node 1 at wk 1. (H) DUN. (I) IM. (J-K) SEM of node 1 at week 0. (J) DUN. (K) IM. (L-M) SEM of node 1 at wk 1. (L) DUN. (M) IM. (N-O) longitudinal sections through node 1 at wk 2. (N) DUN. (O) IM. (P-Q) longitudinal sections through node 1 at wk3. (P) DUN. (Q) IM. (R-S) SEM of node 1 at wk2. (R) DUN. (S) IM. (T-U) SEM of node 1 at wk3. (T) DUN. (U) IM. SAM, shoot apical meristem; L1 and L2, leaves at node 1 and 2 (respectively) of the main axis; MA, main axis; AXM1, primary axillary meristem; AXM2, secondary axillary meristem; P, prophyll; Br, branch. All scale bars are 100  $\mu$ M except inset in (T) is 50  $\mu$ M

These AXMs were approximately the same size, indicating that they initiated at exactly the same time, and were just initiating a first set of leaf buttresses (Fig. 2-1 F-G and J-K). By wk 1, the primary AXM of plants from DUN and IM populations were clearly vegetative, both having prophylls (Fig. 2-1 L and M), forming an axillary bud. AXMs of plants from both population were about the same size and had not begun to initiate a second pair of leaves (Fig. 2-1 H-I and L-M) and were thus at the same developmental stage.

Differences in axillary bud development were first apparent at wk 2 (Fig. 2-1 N-O and R-S). Plants from both populations had initiated a secondary, serial AXM in each leaf axil, proximal to the primary vegetative axillary bud. The distal primary bud on DUN plants was larger than that of IM plants, and had bore multiple leaf pairs (Fig. 2-1 N and R). At wk 2, the prophylls of axillary buds of IM plants had not expanded and no new leaf primordia were initiated. (Fig. 2-1 N and S).

By wk 3, internodes on the primary DUN axillary bud had begun to expand as it grew out into a branch (Fig. 2-1 P and T). In contrast, at wk 3, the primary vegetative bud born on the majority of IM plants had initiated several leaf pairs, but showed no internode expansion (Fig. 2-1 Q and U). At wk 3, the secondary AXMs of both DUN and IM plants had initiated only prophylls (Fig. 2-1 T-U) and the prophylls were roughly the same size.

***At node 2 meristem fate and outgrowth explain differences in branching*** — At Node 2, DUN and IM plants initiated axillary and secondary axillary meristems at similar times. Differences in branch number at node 2 were caused by both differences in axillary meristem fate (always vegetative in DUN plants vs. occasionally floral in IM plant) and the earlier and more frequent outgrowth of DUN vegetative axillary meristems as branches (detailed results presented in appendix K).



***Gene expression analyses*** — Because differences in branch number at node 2 are caused by meristem outgrowth and fate (appendix K), I examined candidate gene expression only at node 1 where meristem outgrowth alone determines branch number (Fig 2-1). *MgMAX1*, 2, 3, and 4 expression differed between DUN and IM populations, and varied among biological samples within populations. *MgMAX* expression for DUN plants was very low at some stages (e.g. DUN *MgMAX3*, Fig. 2C), but was never zero. Because I included three technical replicates within three biological samples for each gene, tissue, and time point assayed, and detected the appropriate *MgMAX* transcripts in all samples, the near zero values for relative *MgMAX* expression do not reflect technical failures during tissue preparation or amplification, but rather reflect actual low relative expression levels. *MgMAX1* expression did not correlate with branching phenotype (appendix L).

*In shoots, MgMAX2, 3, and 4 expression correlates with branching phenotype* At node one, DUN plants expressed one or more of the *MgMAX* genes at significantly lower levels than IM plants throughout development. IM plants never expressed any of the four *MgMAX* genes at significantly lower levels than DUN plants (Fig 2-2; appendix L). DUN plants expressed *MgMAX2* at significantly lower levels than IM plants at week 2 and expressed *MgMAX3* and 4 at significantly lower levels than IM plants at wks 1-4. (Fig 2-2 A).

*Roots, MgMAX3, and 4 expression correlates with branching phenotype* — At week one, there were no significant differences between DUN and IM *MgMAX* expression in root tissue (Fig. 2-2 B). In the top (proximal) half of roots, DUN plants expressed both *MgMAX3*, and 4 at significantly lower levels than IM plants at week 2 (Fig. 2-2 C). In the bottom half of roots,

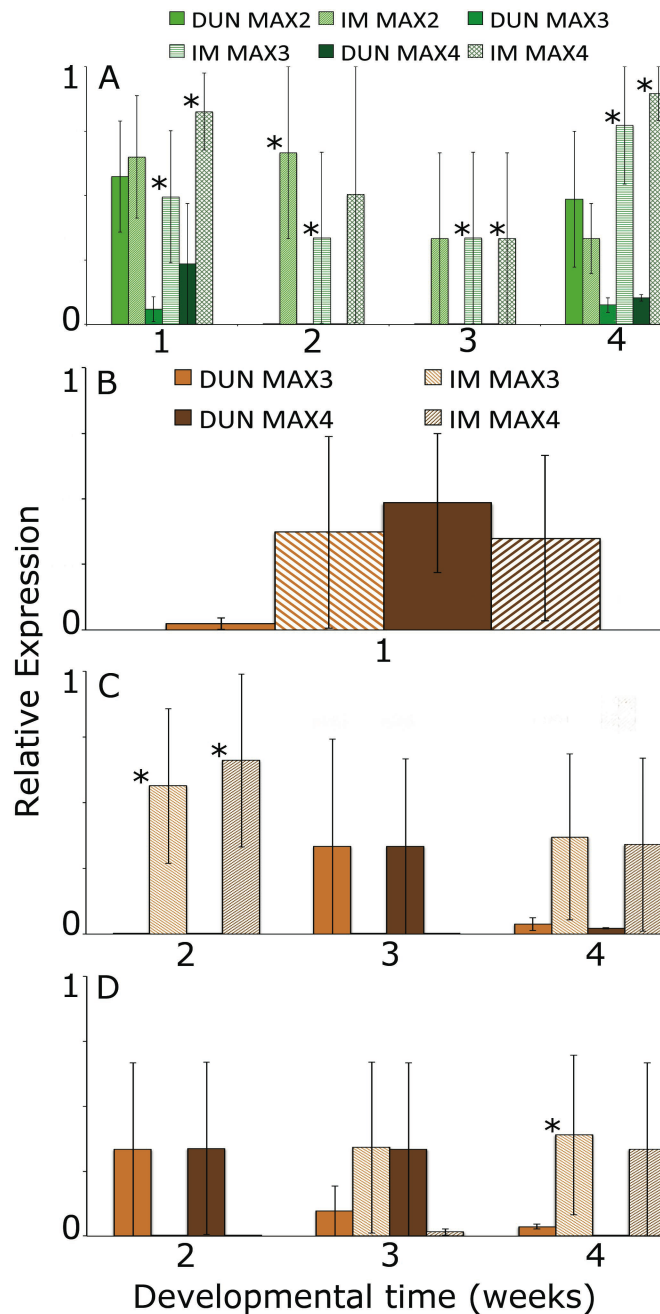


Fig. 2-2 Relative Expression patterns of *Mimulus guttatus* *MORE AXILLARY GROWTH2*, 3, and 4 (*MgMAX2*, 3, and 4) mRNA during development in root and shoot tissue from plants from Dunes and Iron Mountain populations. (A) Shoots (node 1). (B) All roots (week 1). (C) Top half of roots (weeks 2-4). (D) Bottom half of roots (weeks 2-4). Solid bars are Dunes (DUN) plants and striped bars are Iron Mountain (IM) plants. Non-overlapping standard error bars are interpreted as significantly different relative expression levels (indicated with an asterisk) between populations at any single time point (see materials and methods for details).

DUN plants expressed *MgMAX3* at a significantly lower level than IM plants at week 4 (Fig. 2-2 D).

## DISCUSSION

*Mimulus guttatus* from the DUN population are perennials that flower relatively late in development and produce many vegetative branches. In contrast, *M. guttatus* from the IM population are annuals that flower early and have few vegetative branches (Hall and Willis, 2006). Differences in branch number between DUN and IM plants occur primarily at the two basal-most nodes on the main axis (Baker and Diggle, 2011). These differences in branching could potentially result from differences in the timing and number of axillary meristems initiated, frequency of outgrowth, and because vegetative branches and flowers are both products of axillary meristems, differences in meristem fate. In plants from the DUN and IM populations, primary meristems at the first two nodes are initiated at the same time; differences in branch number between plants of the two populations are not caused by differences in the timing or frequency of axillary meristem initiation.

At node 2, branch number is a composite of differences in both meristem fate (solely vegetative in DUN plants, vegetative and floral in IM plants) and vegetative axillary meristem outgrowth (appendix K). At node 1 on the main axis, however, all primary axillary meristems on both DUN and IM plants, regardless of whether they grow out or not, initiate prophylls and are clearly vegetative (Fig. 2-1 A-U). Therefore, differences in branch number at node 1 cannot be attributed to contrasting meristem fate. Instead, population level differences in branch number at node 1 are entirely due to differences in axillary meristem outgrowth vs. quiescence, including onset (earlier in DUN plants) and frequency (greater in DUN plants; Fig. 2-1A and 2-1 N-U)

with which axillary meristems grow out into branches. The differential development of meristems into branches is first detected at week 2; well before differences in branch number are visible at the macromorphological level at week 4.

**MAX expression in *M. guttatus*** — Levels of *MgMAX2*, *3*, and *4* expression are negatively correlated with branch outgrowth in DUN and IM populations of *M. guttatus*. Gene function in the *MAX* pathway is highly conserved: all known *MAX* orthologs in every organism studied to date inhibit branch outgrowth (Napoli and Ruehle, 1996; Stirnberg et al., 2002; Sorefan et al., 2003; Booker et al., 2004; Booker et al., 2005; Snowden et al., 2005; Auldridge et al., 2006; Johnson et al., 2006; Arite et al., 2007; Vogel et al., 2010). Furthermore, knocking out any one of these genes in all model organisms studied thus far results in increased branching. In naturally occurring populations of *M. guttatus* with different branching phenotypes, all instances of significant differences in expression of *MgMAX2*, *3* and *4*, were in the predicted direction: lower levels of *MAX* expression occurred in the relatively highly branched DUN plants, a pattern consistent with the hypothesis that the *MAX* pathway has a role in negative regulation of branching.

In model taxa, *MAX* gene function has been studied in genetically homogeneous lines using mutant screens and reverse genetics. My study populations likely harbored considerable background genetic variation that contributed to substantial variation in branch number (Figs. 2-1A and appendix K) and, presumably, in *MgMAX1*, *2*, *3*, and *4* expression (Figs. 2-2 and appendix L). Despite the variation inherent in natural populations, I observe a consistent pattern of significantly lower expression of *MgMAX3* and *4* in shoots of plants from the branched DUN population than the unbranched IM population throughout early ontogeny. At wk 2, I observe a sharp decrease in *MgMAX2* expression in DUN shoots (relative to IM shoots). At the same time,

I observe a similar decrease in DUN *MgMAX3* and *4* in roots (relative to IM roots). This pulse of decreased *MgMAX2*, *3*, and *4* expression in DUN compared to IM plants occurs at the same time that axillary meristems in DUN plants begin growing out into branches while IM axillary meristems do not and is strong evidence that *MgMAX2*, *3*, and *4* are involved in regulating variation in branch phenotype between to natural and locally adapted populations.

*MAX2*, *3* and *4* are particularly promising candidate genes for understanding natural variation in branching. In *A. thaliana*, *MAX2*, *3* and *4* do not function solely as binary switches regulating branch outgrowth. Instead, QTL studies (*MAX2* and *3*) and heterozygous *MAX3/max3* and *MAX4/max4* mutants with intermediate phenotypes demonstrate the ability of *MAX2*, *3* and *4* to regulate branching in a quantitative, dosage dependent manner (Booker et al., 2004; Auldridge et al., 2006; Ehrenreich et al., 2007). In *M. guttatus* branch number varies both between and within DUN and IM populations, indicating that the mechanisms responsible for controlling natural variation in *M. guttatus* branch growth must act in a dosage-dependent manner similar to that in *A. thaliana*. Furthermore, while *AtMAX3* and *4* (and all their known orthologs) produce a hormone that functions at the whole-plant level, *AtMAX2* (and all its known orthologs) functions only in shoots to inhibit branch outgrowth, and it does so specifically at each node and in close association with each axillary bud (Stirnberg et al., 2007). If *MgMAX2* functions similarly to *AtMAX2* (and all known *MAX2* orthologs), it may provide a mechanisms to differentially regulate the amount of shoot branching along the main axis.

*Gene expression and secondary axillary meristems* — Regulation of branch outgrowth is a complex and multigenic phenomenon (Aguilar-Martinez et al., 2007; Finlayson et al., 2010) and *MgMAX1*, *2*, *3*, and *4* clearly are not solely responsible for regulating outgrowth of vegetative axillary meristems as branches. As evidence of this, DUN and IM plants both

initiated secondary vegetative axillary meristems. Regardless of *MgMAX1*, 2, 3, and 4 expression levels, these secondary meristems remained quiescent in both populations. Additional pathways may control differences between primary and secondary axillary meristem outgrowth within a single axil. For instance, the strigolactone signal generated by *AtMAX1*, 3, and 4 appears to be conducted through the plant vasculature. The vasculature of the primary axillary meristems in *M. guttatus* is well differentiated at the time the meristems begin to develop into branches. In contrast, the associated secondary axillary meristems lack differentiated conducting tissues. Perhaps the differential development of the vascular tissue plays a role in contrasting regulation of primary and secondary axillary meristems. Alternatively, or in addition, more precise regulation of *MgMAX2* specific to the individual meristem may control secondary vs. primary axillary meristem outgrowth. Detailed QTL studies of branching in *M. guttatus* would likely reveal additional loci involved in outgrowth of primary axillary meristems, however, because variation in secondary axillary meristem outgrowth has not been identified, QTL studies cannot determine the loci that regulate secondary axillary meristem outgrowth. Regardless of the role of additional loci, *MgMAX2*, 3, and 4 represent strong candidates for future functional studies that validate the role of developmental genes in *M. guttatus* branching.

**Conclusion** — In order to fully understand the evolution of plant development, it is necessary to examine developmental genetic pathways that control well-characterized and evolutionarily relevant morphological variation within and among populations, where genetic divergence, adaptation, and speciation occur (Cresko et al., 2007; Johnson, 2007). I link gene expression to functional phenotypes through molecular and morphological development in natural populations of *M. guttatus*. Specifically, I demonstrate that the *MAX* pathway, which is conserved across at least 150 million years of evolutionary history and is

integral to plant body plan development, is strongly associated with branch number. I provide some of the first quantitative molecular genetic data from plants that associates candidate genes with intraspecific, natural variation in a functionally and evolutionarily central aspect of morphology in two locally adapted populations over the course of development. Branch development is an important part of plant function and life history evolution (Geber, 1990; Baker and Diggle, 2011). Trade-offs between branching and flowering can influence flowering time (Geber, 1990; Zopfi, 1995a; Bonser and Aarssen, 1996; Bonser and Aarssen, 2006), including in *M. guttatus* where flowering time and branch number are negatively correlated (Hall et al., 2006; Baker and Diggle, 2011). Flowering time is a critical component of local adaptation in DUN and IM populations (Hall and Willis, 2006). Because of the tight relationship between flowering and branching (Zopfi, 1995b; Bonser and Aarssen, 1996; Prati and Schmid, 2000; Bonser and Aarssen, 2006), branching likely also contributes to local adaptation in these populations.

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## APPENDICES

### APPENDIX A

Results for model tests for all statistical models used. Y is the dependent variable; the independent variables include population (Pop) and week (Wk), and their interaction terms. Random variables tested include unique plant ID (UID), nested within family. When there was no significant difference between models, the simplest model (model B) was used. Significant differences are denoted with an asterisk. When models A and B were significantly different, the model with the lowest AIC (bold) score was used. AIC is the Aikeke Information Criterion and likelihood values are presented in log form. The percent of the variation explained by each random effect used in the best model is given.

Model A:  $Y \sim \text{Pop} * \text{Wk}$ , random= $\sim 1 | \text{family/UID}$

Model B:  $Y \sim \text{Pop} * \text{Wk}$ , random= $\sim 1 | \text{UID}$

Dependent Variable (Y)	Model A (AIC; likelihood)	Model B (AIC; likelihood)	Variation explained by Family	Variation explained by ID
Height (cm)	<b>-12.76; 13.380</b>	3.04; 4.47*	20.0%	51.8%

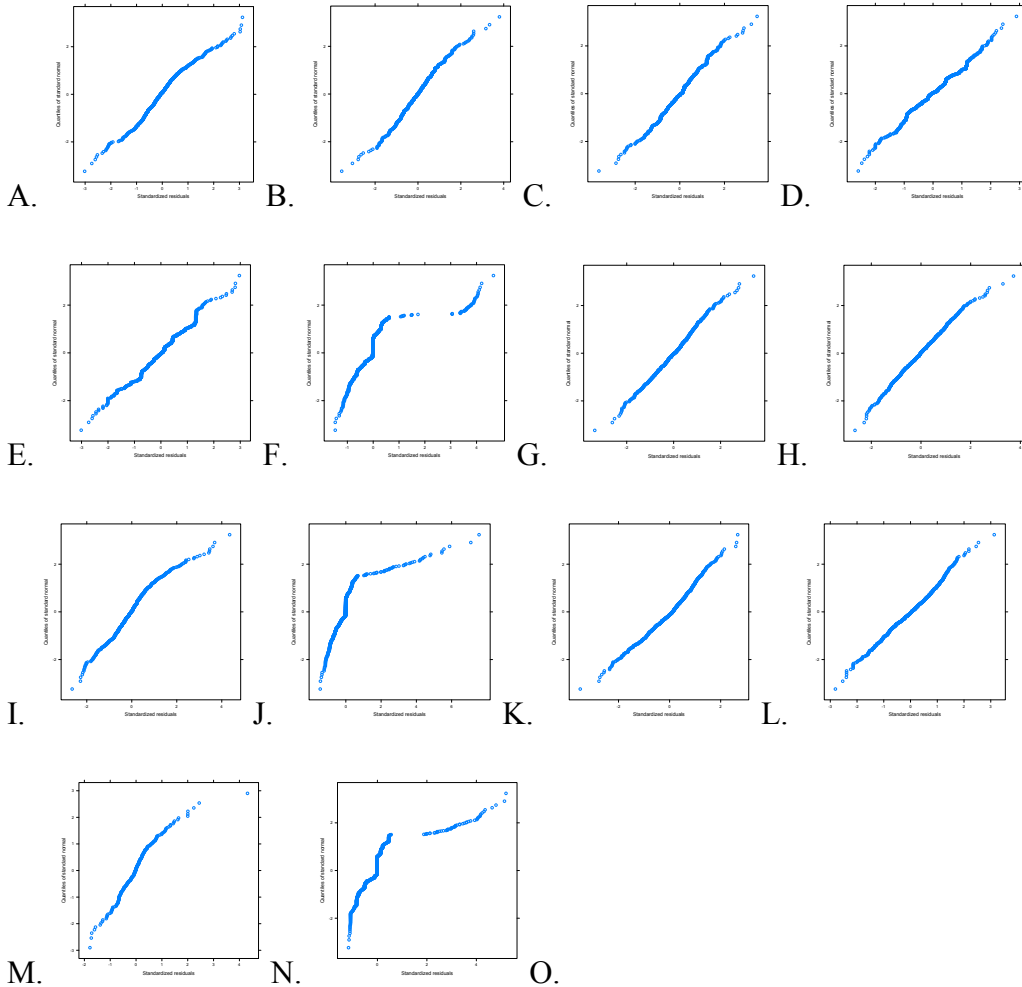
Dependent Variable (Y)	Model A (AIC; likelihood)	Model B (AIC; likelihood)	Variation explained by Family	Variation explained by ID
Height (# of nodes)	<b>1833.95; 1866.89</b>	1847.41; 1875.64*	17.3%	42.0%
Total Branch #	2549.79; -1267.89	2547.84; -1267.92	n/a	38.7%
N1 Branch #	1672.84; -829.42	1672.02; -830.01	n/a	35.3%
N2 Branch #	1523.90; -754.95	1521.90; -754.95	n/a	30.9%
N3 Branch #	<b>941.08; -463.54</b>	950.64; -469.32*	4.49%	0.0%
Total Branch length (cm)	1839.97; -912.98	1840.48; -914.24	n/a	0.0%
N1 Branch length (cm)	1640.34; -813.17	1642.11; -815.05	n/a	61.11%
N2 Branch length (cm)	1618.87; -801.43	1618.82; -803.41	n/a	75.3%
N3 Branch length (cm)	<b>-1270.76; 642.38</b>	-1262.86; 637.43*	0.5%	0.9%
Total nodes on branches	1641.46; -814.10	1641.46; -814.73	n/a	38.5%
N1 nodes on branches	1422.33; -704.16	1423.07; -705.53	n/a	37.4%

Dependent Variable (Y)	Model A (AIC; likelihood)	Model B (AIC; likelihood)	Variation explained by Family	Variation explained by ID
N2 nodes on branches	2160.60; - 1073.301	2158.77; -1073.38	n/a	31.8%
N3 nodes on branches	<b>430.65; -208.32</b>	440.39; 440.39*	4.2%	0.0%

*Notes:* Total Branch # includes branches at nodes 1-4.

## APPENDIX B

Quantile-quantile plots of the residuals the best model (Appendix 1) for each statistical test. (A) Main axis height. (B) Number of nodes on the main axis. (C) Number of branches at nodes 1-4. (D) Number of branches at node 1. (E) Number of branches at node 2. (F) Number of branches at node 3. (G) Total branch length at nodes 1-4. (H) Total branch length at node 1. (I) Total branch length at node 2. (J) Total branch length at node 3. (K) Number of nodes on branches at nodes 1-4 of the main axis. (L) Number of nodes on branches at node 1 of the main axis. (M) Number of nodes on branches at node 2 of the main axis. (N) Number of nodes on branches at node 3 of the main axis. Note that all Node 3 plots (F, J, and N) deviate from normal.



# APPENDIX C

Mean values for branch number for *Mimulus guttatus* plants derived from the Dunes and the Iron Mountain populations.

Total				Node 1		Node 2		Node 3		Node 4	
DUN	n	Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI
Week											
1	72	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	134	0.17	0.09	0.17	0.09	0.00	0.00	0.00	0.00	0.00	0.00
3	62	0.55	0.21	0.55	0.21	0.00	0.00	0.00	0.00	0.00	0.00
4	131	2.70	0.28	1.47	0.14	1.19	0.16	0.04	0.04	0.00	0.00
5	129	3.44	0.30	1.55	0.13	1.53	0.14	0.35	0.13	0.02	0.03
6	59	3.95	0.41	1.49	0.20	1.64	0.18	0.78	0.24	0.03	0.07
IM											
Week											
1	26	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	63	0.05	0.05	0.03	0.04	0.02	0.03	0.00	0.00	0.00	0.00
3	34	0.47	0.32	0.26	0.19	0.15	0.19	0.06	0.12	0.00	0.00
4	46	0.83	0.29	0.52	0.21	0.30	0.19	0.00	0.00	0.00	0.00
5	50	1.22	0.31	0.94	0.27	0.28	0.16	0.00	0.00	0.00	0.00
6	20	1.75	0.65	0.90	0.37	0.70	0.38	0.00	0.00	0.15	0.21

Notes: DUN, dunes; IM, Iron Mountain; n, sample size; CI, confidence interval; Total includes nodes 1-4.



## APPENDIX D

Natural log transformed means and confidence intervals for branch length (in cm) from the Dunes and Iron Mountain *Mimulus guttatus* populations.

		Total		Node 1		Node 2		Node 3		Node 4	
DUN	n	Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI
Week											
1	72	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	134	0.02	0.01	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00
3	62	0.04	0.02	0.04	0.02	0.00	0.00	0.00	0.00	0.00	0.00
4	131	0.41	0.05	0.30	0.04	0.15	0.03	0.00	0.00	0.00	0.00
5	129	0.64	0.07	0.37	0.05	0.36	0.05	0.04	0.02	0.00	0.00
6	59	0.77	0.11	0.36	0.07	0.46	0.09	0.15	0.06	0.01	0.02
IM											
Week											
1	26	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	63	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3	34	0.09	0.08	0.02	0.02	0.06	0.08	0.01	0.01	0.00	0.00
4	46	0.20	0.10	0.08	0.04	0.12	0.09	0.00	0.00	0.00	0.00
5	50	0.44	0.14	0.30	0.11	0.16	0.12	0.00	0.00	0.00	0.00
6	20	0.59	0.26	0.33	0.18	0.23	0.19	0.00	0.00	0.11	0.16

*Notes:* DUN, dunes; IM, Iron Mountain; n, sample size; CI, confidence interval; Total includes nodes 1-4.

## APPENDIX E

Means and confidence limits for back-transformed values for branch length (in cm) for plants from the Dunes and Iron Mountain populations of *Mimulus guttatus* at Nodes 1-4 (Total), Node 1, and Node 2.

		Total			Node 1			Node 2		
DN Wk	n	Mean	Upper	Lower	Mean	Upper	Lower	Mean	Upper	Lower
			95%	95%		95%	95%		95%	95%
			limit	limit		limit	limit		limit	limit
1	72	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	134	0.02	0.03	0.01	0.02	0.03	0.01	0.00	0.00	0.00
3	62	0.04	0.02	0.02	0.04	0.06	0.02	0.00	0.00	0.00
4	131	0.50	0.43	0.08	0.35	0.40	0.29	0.17	0.20	0.13
5	129	0.89	1.03	0.76	0.45	0.53	0.38	0.43	0.50	0.36
6	59	1.15	1.41	0.92	0.43	0.52	0.33	0.59	0.74	0.45
IM Wk										
1	26	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	63	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3	34	0.09	0.19	0.01	0.02	0.06	0.00	0.06	0.15	-0.02
4	46	0.22	0.34	0.11	0.09	0.14	0.04	0.12	0.23	0.02
5	50	0.55	0.78	0.34	0.35	0.50	0.21	0.17	0.31	0.04
6	20	0.81	1.34	0.40	0.39	0.65	0.16	0.25	0.52	0.04

*Notes:* DN, dunes; IM, Iron Mountain; n, sample size; CI, confidence interval; Total includes nodes 1-4. Upper and lower limits are 95% confidence limits. Both upper and lower limits are given because the upper and lower back transformed confidence intervals are unequal

Means and confidence limits for back-transformed values for branch length (in cm) for plants from the Dunes and Iron Mountain populations of *Mimulus guttatus* at Node 3 and Node 4.

Node 3					Node 4		
DUN Wk	N	Mean	Upper 95% limit	Lower 95% limit	Mean	Upper 95% limit	Lower 95% limit
1	72	0.00	0.00	0.00	0.00	0.00	0.00
2	134	0.00	0.00	0.00	0.00	0.00	0.00
3	62	0.00	0.00	0.00	0.00	0.00	0.00
4	131	0.00	0.01	0.00	0.00	0.00	0.00
5	129	0.04	0.06	0.03	0.00	0.00	0.00
6	59	0.16	0.23	0.09	0.01	0.02	0.00
IM Wk							
1	26	0.00	0.00	0.00	0.00	0.00	0.00
2	63	0.00	0.02	0.00	0.00	0.00	0.00
3	34	0.01	0.01	0.01	0.00	0.00	0.00
4	46	0.00	0.00	0.00	0.00	0.00	0.00
5	50	0.00	0.00	0.00	0.00	0.00	0.00
6	20	0.00	0.00	0.00	0.12	0.31	0.00

*Notes:* DN, dunes; IM, Iron Mountain; N, sample size; CI, confidence interval. Upper and lower limits are 95% confidence limits. Both upper and lower limits are given because the upper and lower back transformed confidence intervals are unequal.

## APPENDIX F

Means for back-transformed values for branch length (nodes per branch) for *Mimulus guttatus* plants from the Dunes and Iron Mountain populations.

		Total			Node 1			Node 2	
DUN	n	Mean	Upper 95% limit	Lower 95% limit	Mean	Upper 95% limit	Lower 95% limit	Mean	Upper 95% limit
Week									
1	72	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	134	0.02	0.04	0.01	0.02	0.04	0.01	0.00	0.00
3	62	0.17	0.32	0.07	0.17	0.32	0.07	0.00	0.00
4	131	2.37	2.82	1.96	1.38	1.66	1.34	0.76	0.97
5	129	3.61	4.12	3.13	1.76	2.07	1.47	1.29	1.53
6	59	4.49	5.29	3.76	1.55	2.01	1.17	1.78	2.21
IM Week									
1	26	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	63	0.00	0.01	0.00	0.00	0.01	0.00	0.00	0.00
3	34	0.18	0.46	0.03	0.08	0.21	0.01	0.03	0.11
4	46	0.70	1.20	0.33	0.32	0.61	0.12	0.11	0.29
5	50	1.68	2.53	1.01	1.07	1.74	0.56	0.14	0.03
6	20	2.84	4.97	1.30	1.39	2.66	0.53	0.71	0.16

*Notes:* DUN, dunes; IM, Iron Mountain; n, samples size; CI, confidence interval; Total includes nodes 1-4. Upper and lower confidence limits are given for back transformed data because the back transformations yield unequal upper and lower confidence intervals.

Means for back-transformed values for branch length (nodes per branch) for *Mimulus guttatus* plants from the dunes and Iron Mountain populations.

		Node 3				Node 4	
DUN	n	Lower	Mean	Upper 95%	Lower 95%	Mean	95% CI
Week		95% limit		limit	limit		
1	72	0.00	0.00	0.00	0.00	0.00	0.00
2	134	0.00	0.00	0.00	0.00	0.00	0.00
3	62	0.00	0.00	0.00	0.00	0.00	0.00
4	131	0.57	0.00	0.00	0.00	0.00	0.00
5	129	1.06	0.06	0.12	0.03	0.02	0.03
6	59	1.39	0.36	0.60	0.18	0.03	0.07
IM Week							
1	26	0.00	0.00	0.00	0.00	0.00	0.00
2	63	0.00	0.00	0.00	0.00	0.00	0.00
3	34	0.00	0.00	0.02	0.00	0.00	0.00
4	46	0.02	0.00	0.00	0.00	0.00	0.00
5	50	0.14	0.00	0.00	0.00	0.00	0.00
6	20	0.71	0.00	0.00	0.00	0.25	0.40

*Notes:* DUN, Dunes; IM, Iron Mountain; n, samples size; CI, confidence interval. Upper and lower confidence limits are given for back transformed data because the back transformations yield unequal upper and lower confidence intervals. Data for node 4 did not require transformation.

## APPENDIX G

Means for back transformed (cm) and raw data (nodes) for plant height from plants derived from the dunes and Iron Mountain populations of *Mimulus guttatus*.

Back-transformed height (cm)					Number of Nodes of the main axis	
DUN			Upper 95% CI	Lower 95%		
Week	n	Mean	limit	CI limit	Mean	95% CI
1	72	0.58	0.63	0.53	2.68	0.12
2	134	0.58	0.63	0.53	2.96	0.05
3	62	0.49	0.45	0.53	3.50	0.15
4	131	0.97	0.90	1.04	4.80	0.12
5	129	1.46	1.35	1.60	5.71	0.16
6	59	1.38	1.21	1.54	6.02	0.21
IM Week						
1	26	0.46	0.34	0.57	2.46	0.22
2	63	0.92	0.75	1.08	3.30	0.20
3	34	1.05	0.87	1.21	3.85	0.28
4	46	2.82	2.17	3.38	5.33	0.41
5	50	3.78	3.03	4.43	6.27	0.40
6	20	3.47	2.72	4.11	6.20	0.63

*Notes:* DUN, dunes; IM, Iron mountain; n, sample size; CI, confidence interval. Upper and lower confidence limits are given for back transformed data (height in cm) because upper and lower back transformed confidence intervals are unequal.

## APPENDIX H

Flowers on the main axis of plants from the Iron Mountain population of *Mimulus guttatus*.

Wk	N	Node	95%	Node	95%	Node	95%	Node	95%	Nodes	95%	Nodes	95%
		1	CI	2	CI	3	CI	4	CI	1-4	CI	≥5	CI
1	26	0.00	0.00	0.19	0.19	0.04	0.08	0.00	0.00	0.23	0.20	0.00	0.00
2	63	0.03	0.06	0.32	0.15	0.44	0.18	0.05	0.07	0.84	0.29	0.00	0.00
3	34	0.00	0.00	0.12	0.14	0.50	0.19	0.18	0.15	0.79	0.26	0.00	0.00
4	46	0.00	0.00	0.48	0.22	0.98	0.22	0.80	0.26	2.26	0.46	1.46	0.56
5	50	0.00	0.00	0.42	0.19	1.08	0.20	1.16	0.21	2.66	0.45	2.50	0.60
6	20	0.00	0.00	0.10	0.13	0.85	0.33	1.10	0.31	2.05	0.52	2.25	0.94

*Notes:* Wk, week; N, sample size; CI, confidence interval.

# APPENDIX I

Table 1. Flowers on the main axis of *Mimulus guttatus* plants from the Dunes population.

Wk	n	Node 1	95% CI	Node 2	95% CI	Node 3	95% CI	Node 4	95% CI	Node 1-4	95% CI	Node $\geq 5$	95% CI
1	72	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	134	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3	62	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4	131	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.04	0.05	0.04	0.05	0.04
5	129	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.04	0.05	0.04	0.43	0.13
6	59	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.44	0.20

Notes: Wk, week; n, sample size; CI, confidence interval.



## APPENDIX J

**Detailed molecular genetic methods – Candidate gene identification** – Four genes in the *Arabidopsis thaliana* MORE AXILLARY GROWTH (*AtMAX*) pathway constitute the most thoroughly characterized molecular mechanism for regulating axillary meristem repression and outgrowth in eudicots. When any one of these genes is inactivated, the plant develops substantially more branches than wildtype *Arabidopsis* (Bainbridge et al., 2005; Booker et al., 2004; Stirnberg et al., 2002). *MAX* orthologs have been characterized in other model species such as *Pisum sativum*, *Petunia hybrida*, and *Oryza sativa* as well as the non-model organism *Actinidia chinensis* (kiwifruit) (Johnson et al., 2006; Ledger et al., 2010; Snowden et al., 2005; Sorefan et al., 2003; Zou et al., 2006).

In *A. thaliana* there are four single copy genes in the *MAX* pathway: *AtMAX1* is a cytochrome p450, *AtMAX2* is an F-box gene, and *AtMAX3* and *AtMAX4* are carotenoid cleavage dioxygenases. *Arabidopsis AtMAX 1, 2, 3, and 4* were used as query sequences in tBLASTx searches of the *M. guttatus* genome (Altschul et al., 1997). Matches with E values  $< 1 \times 10^{-50}$  were considered putative *M. guttatus MAX* orthologs. In the case of *AtMAX2*, *AtMAX3*, and *AtMAX4*, there was only one putative *M. guttatus* ortholog for each *A. thaliana* gene (hereafter, *MgMAX2*, *MgMAX3*, and *MgMAX4*). There were two putative *M. guttatus MAX1* orthologs. Based on identical scores and E-values, nucleotide sequence similarity (>99%, including >99% similarity in putative introns), and the location of one putative ortholog on a small, potentially orphan scaffold (scaffold 853), these sequences likely represent the same gene and do not reflect a duplication of *MAX1* in *M. guttatus* (hereafter, *MgMAX1*).

**Candidate gene sequence confirmation** –The cDNA sequences of all four *MgMAX1, 2, 3,* and *4* were confirmed in plants derived from both the DUN and IM populations of *M. guttatus*

using the DUN10 and IM62 (the line used for the genome sequence) lines provided by the *Mimulus* seed stock center at Duke University. RNA was extracted from the shoot apices of one DUN10 and one IM62 plant using RNeasy mini kits and treated with DNaseI following the manufacturer's protocols (Qiagen). DNase treated RNA was used to generate cDNA using SuperScript III (Invitrogen) reverse transcriptase and random hexamer primers according to the manufacturer's protocols. Gene specific primers designed to amplify 500 bp segments of each of the candidate genes were based off of the draft *M. guttatus* genome sequence. PCR was performed using the Expand High Fidelity PCR system (Roche) following the manufacturer's instructions using a gradient of 42 to 65<sup>0</sup>C and 40 cycles on an Eppendorf Mastercycler Gradient thermocycler. PCR products of expected size were gel extracted from three independent reactions for both DUN and IM cDNA templates (Qiagen Gel Extraction Kit) and cloned into the pCR 4-TOPO TA cloning vector, which was transformed into alpha-DH5 chemically competent cells (Invitrogen) following the manufacturer's protocols and screened using carbenicillin. Plasmids were isolated from ten positive clones per PCR reaction using the Plasmid Mini Kit following the manufacturer's instructions (Qiagen). Sequencing reactions were performed using M13F and M13R primers by Functional Biosciences. Vector and primer sequences were trimmed and base calls confirmed by visual inspection of chromatograms in Sequencher v4.6-4.10 (Gene Codes).

*Tissue collection – MAX* expression in *Arabidopsis*, *Pisum*, and *Petunia* is not restricted to shoots. The highest levels of *AtMAX1*, 3 and 4 (and their respective orthologs in *Pisum*, *Petunia*, *Oryza*, and *Actinidia*) function in roots, where these genes function (Booker et al., 2004; Johnson et al., 2006; Snowden et al., 2005; Sorefan et al., 2003; Zou et al., 2006). *AtMAX1*, 3 and 4 (and their orthologs in other species) are part of a pathway that synthesizes an upwardly

mobile strigolactone hormone signal (Gomez-Roldan et al., 2008; Schwartz et al., 2004; Umehara et al., 2008)(Beveridge et al., 1996; Johnson et al., 2006). *AtMAX1*, 3, and 4 also function in shoots. *AtMAX2* (and its ortholog in *Pisum*) is expressed in the shoot at nodes where it perceives the hormone generated by *AtMAX3*, *AtMAX4*, and *AtMAX1*, and negatively regulates branch outgrowth (Stirnberg et al., 2007).

Because a subset of *MAX* genes function in roots, I collected tissue from both roots and shoots. I assayed *MgMAX1*, 2, 3, and 4 expression at node 1 in DUN and IM plants and *MgMAX1*, 3 and 4 in roots of DUN and IM plants. I did not assay *MgMAX1* or *MgMAX2* expression at node 2 because at node 2 axillary meristem fate (floral vs. vegetative in IM plants) contributes to differences in branch number. It is unclear whether *MAX* genes are involved in floral meristem outgrowth and I did not want to confound my results concerning branch outgrowth with the additional variable of meristem fate. Therefore, node 1 (where all meristems in both populations were vegetative) is a much more promising location for examining *MgMAX* expression.

All tissue collected was staged to correspond to previously published data on the morphological development of branches in DUN and IM populations of (Fig. 1A; adapted from Baker and Diggle, 2011) and samples from this study used for histology and SEM. Tissue was collected from two maternal families from each population that represented the most extreme branching phenotypes typical of each population (Baker and Diggle, 2011). Tissue was collected from 15 plants per population per time point. Tissue from 2-5 plants per population per time point was pooled into a single biological sample. Biological samples were replicated three times for each population, time point, and category of tissue collected. Shoot tissue was collected by quickly dissecting out node 1 of main axis of DUN and IM plants. Subtending leaves were

trimmed away but all axillary products were left intact. Roots were quickly cleaned of all substrate under cold water. Roots from each plant were divided into two samples: 1) the lower half (R2): from the distal end of the root mass to half the length of the roots and 2) upper half (R1): from base of the hypocotyl to half way down the length of the root mass because in *Petunia*, *DECREASED APICAL DOMINANCE1* (*dad1*), the *Petunia* *MAX4* ortholog, expression decreases from distal to proximal root tissue (Snowden et al., 2005). At week one, all root tissue was collected in a single sample (Ra). All tissue was immediately frozen in liquid nitrogen and stored at -80°C.

*Sample Preparation* – RNA was extracted from 10-100mg of tissue from each sample using RNeasy mini RNA extraction kits according to the manufacturer's protocol (Qiagen). All RNA samples were treated with TURBO DNA-free DNase (Ambion) and RNA concentration was verified with a Qubit 2.0 fluorometer (Invitrogen). A subset of extractions was concentrated for 20 min at room temperature in a vacufuge (Eppendorf). RNA quality was assessed using an Agilent 2100 BioAnalyzer; samples with strong 26S and 18S ribosomal RNA peaks that also did not exhibit degradation were reverse transcribed into cDNA (+RT) using 1.0 µg RNA and an iScript reverse transcriptase kit (including random primers) according to the manufacturer's protocol (BioRad). Ten randomly selected DNase-treated RNA samples were used for additional cDNA synthesis reactions without reverse transcriptase (-RT). Actin primers (that do not span an intron) were used in quantitative RT PCR (protocol described below) with +RT and -RT templates in tandem (Scoville et al., 2011). Amplification from +RT cDNA but not -RT samples indicated successful cDNA synthesis and absence of contaminating genomic DNA.

*Quantitative reverse transcription PCR (qRT-PCR)* — All qRT-PCR reactions were performed on an Agilent Technologies Mx3005P thermocycler (Stratagene) using 1.0µL of

cDNA template, diluted 1:10 in water, Brilliant III Ultra Fast SYBR Green QPCR Master Mix (Agilent Technologies) with a final concentration of 1x, and a final concentration of 500 nM for all primers. All reactions were performed using a Master Mix from a single lot.

Gene specific primer pairs for each candidate gene were designed using Primer3 (v0.4) software to amplify short (100-300 bp) DNA segments. Primers were designed based on inclusive DUN and IM consensus sequences to bind without mismatch to coding regions that are conserved between the populations but otherwise represent unique sequences within the genome, thereby minimizing potential population biases in the reactions. PCR amplicons spanned putative introns when possible (*MgMAX1*, 3, and 4; *MgMAX2* lacks introns). Six sets of primer sequences for five housekeeping reference genes (one primer pair for ubiquitin (Ubq5), actin (Act), 26S ribosomal RNA (26S), and ribosomal binding protein L2 (L2), and two primer pairs for elongation factor 1 $\alpha$  (EF1 $\alpha$ , EF1 $\alpha$ 2)) previously identified from the *M. guttatus* draft genome {Scoville, 2011 #702} were also evaluated. Gene specific primer sequences used were as follows:

*MgMAX1* forward: 5'-AGAAGAAATCGACTCGTTTGC-3',

*MgMAX1* reverse: 5'-TTTCTCCGGTTCTTGGAAT-3';

*MgMAX2*: forward: 5'-GGGCTGAGGAACTGTTCAT-3',

*MgMAX2* reverse: 5'-CCGACCTCATCTCAGTGCT-3';

*MgMAX3* forward: 5'-CAATGCCGAAGATATGTTACTCC-3',

*MgMAX3* reverse: 5'-ATCCGTAAAGGCCCAATCAT-3'; and

*MgMAX4* forward: 5'-GACGTTCCATTTCATCAACG-3' and

*MgMAX4* reverse: 5'-TGCACATATCCATGCCTTTT-3'.

Optimal annealing temperatures and reaction efficiencies were established for primers. Each housekeeping gene primer pair was amplified using a gradient of annealing temperatures from 52 to 62°C in triplicate. Primers that did not amplify a single target (Ubq5 and EF1α2) as assessed by dissociation curves without a single, clean peak were not considered for further evaluation. All remaining housekeeping primers amplified a single product consistently at 60°C, and all future amplifications were conducted with a 60°C annealing temperature. All gene-of-interest primers also consistently produced a single amplicon with a 60°C annealing temperature. Reaction efficiencies were established for the remaining primers for housekeeping genes and all primers for genes of interest by conducting qRT PCR using four separate serial dilutions of (1:10, 1:100, 1:1,000, 1:10,000) of the same cDNA template in triplicate. Reaction efficiencies were calculated by fitting a line to the number of cycles (c(T)) and vs. log(relative quantity) using MxPro QPCR Software (Stratagene); reactions that failed or amplified inconsistently were not included in the calculations. Efficiencies (E) for each qPCR were calculated the equation  $E = 10^{-1/\text{slope}}$  (Rasmussen, 2000). All reaction efficiencies were between 94% and 106%. All efficiencies were treated as less than or equal to 100% for calculations of relative gene expression.

The housekeeping genes with the most stable expression patterns were determined by amplifying DNA from a subset of DUN and IM cDNA samples representing roots, shoots, and the two most extreme developmental time points. qRT PCR was conducted in triplicate and the c(T) values were converted to relative quantities for outlier analysis using the geNORM visual basic application (Vandesompele et al., 2002).

I assessed relative expression levels of *MgMAX1* and *MgMAX2* in tissue from node 1 and relative expression levels of *MgMAX3* and *MgMAX4* in tissue samples of roots. For each time

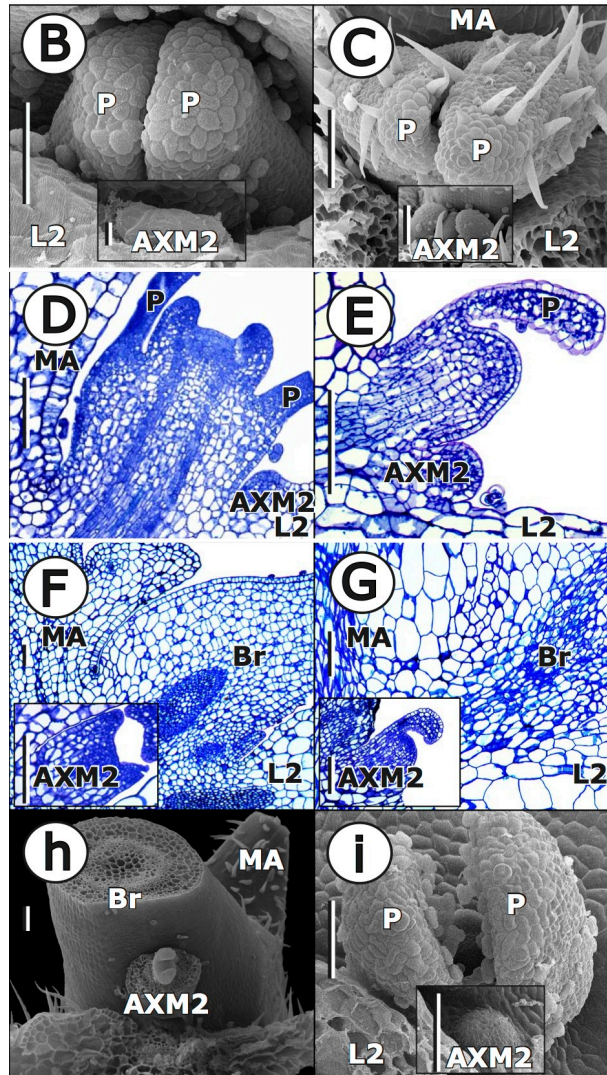
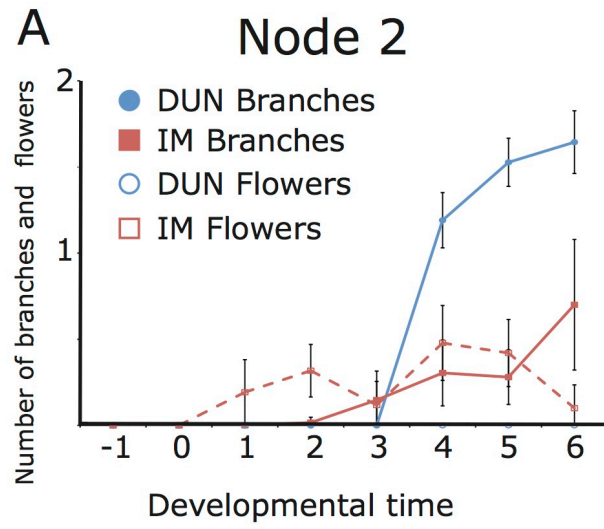
point and tissue type, I performed qRT PCR for four genes (either *MgMAX1* and *MgMAX2* or *MgMAX3* and *MgMAX4* and the two most stably expressed reference genes, EF1 $\alpha$  and Actin) on three biologically samples from the DUN and IM populations in triplicate on a single plate along with no template negative controls for each primer pair, in triplicate. The critical threshold levels were set automatically for each of the eleven plates (four plates for node 1 tissue and 8 plates for the three types of root tissue, Ra (1 plate), and R1 and R2 (three plates each)).

I calculated relative gene-of-interest expression levels normalized with the geometric mean of the two most stable reference genes (EF1 $\alpha$  and Actin) following the *delta c(T)* method described in Scoville et al (2011). Samples that had evidence of spurious amplification (indicated by multiple peaks in the dissociation curves or incorrect melting temperatures) were excluded from the study. Samples that failed to amplify were also excluded from the study, unless all three technical replicates from the same sample failed to amplify, which indicated a relative quantity of zero. For each gene of interest, time-point, and tissue type I calculated relative expression levels by subtracting the minimum critical threshold (*c(T)*) value of each gene from the *c(T)* value of each reaction to generate *delta c(T)* values. Relative expression quantities were calculated by raising the specific efficiency of each primer pair (*E*) to the *-delta c(T)* of each reaction. The relative quantity of the triplicates was averaged to produce a mean relative quantity for each gene, tissue type, time point, and biological replicate. The relative quantity of each biological replicate was normalized by dividing by the geometric mean of the two reference genes for that replicate (Vandesompele et al., 2002). For each gene of interest, tissue type, and time point, normalized expression levels were divided by the sample with the highest normalized expression level, thereby setting the expression level in the sample with the highest expression to 1 for ease of interpretation (Scoville et al., 2011). Comparisons within a given tissue type and

time point do not require adjustment for inter-plate variability because they were all run on a single plate. Relative expression levels were assessed graphically and non-overlapping standard errors were considered evidence of significant differences at  $< 0.05$  level (Cumming et al., 2007).



# Appendix K

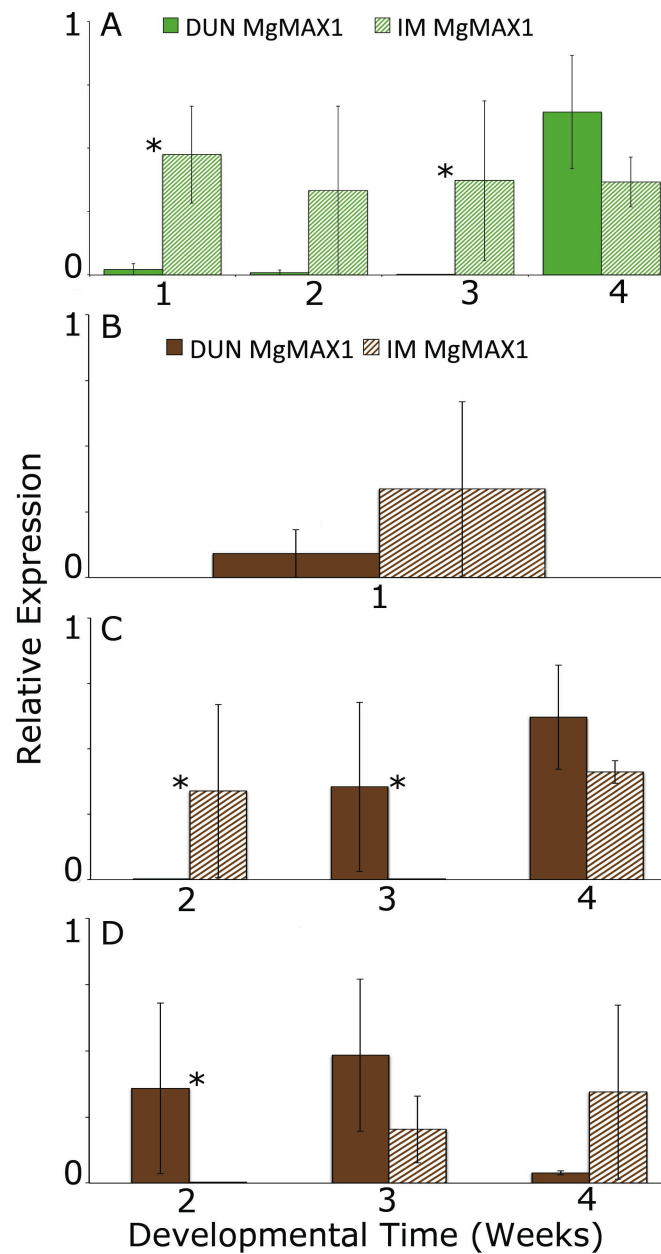


Appendix K. Light and scanning electron microscopy of node 2 in Dunes and Iron Mountain populations of *Mimulus guttatus*. At node 2, IM plants produce branches and occasionally flowers; DUN plants produce only branches and significantly more of them than IM plants (A; adapted from Baker and Diggle, 2011). (B-C) SEM of node 2 at wk 2. (B) DUN. (C) IM. (D-E) longitudinal sections through node 2 at wk 3. (D) DUN. (E) IM. (F-G) longitudinal sections through node 2 at wk 4. (F) DUN. (G) IM. (H-I) SEMs of node 2 at wk 4. (H) DUN. (I) IM. L2, leaves at node 2 of the main axis; MA, main axis; AXM1, primary axillary meristem; AXM2, secondary axillary meristem; P, prophyll; Br, branch. All scale bars are 100  $\mu$ M.

***Meristem initiation, fate, and outgrowth at node 2*** – The morphological patterns of branch and flower development for DUN and IM plants at node 2 is shown in Fig. 2-1 A (adapted from Baker and Diggle, 2011). When cotyledons were first visible above the ground (germination; wk -1), histological and SEM examination of plants from the DUN and IM populations demonstrated that plants from the DUN and IM populations had already initiated a second set of leaf primordia, but lacked meristems in the axils of these primordia (Fig. 2-1 D and E). By wk 0, plants from both populations had initiated a single meristem in the axil of each leaf. It was not until wk 2 that the AXMs of plants from both populations initiated prophylls and became axillary buds (Appendix K, B-C). Plants from both populations also initiated a secondary, serial AXM proximal to the primary axillary bud by wk 2 (Appendix K, B and C, see insets). At wk 3, the primary axillary bud on DUN plants had initiated three leaf pairs whereas the primary vegetative axillary bud on IM plants bore only unexpanded prophylls (Appendix K, D-E). By wk 4, internodes of the primary vegetative axillary bud of DUN plants had expanded, resulting in an axillary branch (Appendix K, F and H). On some IM plants, primary vegetative axillary buds also grew out into branches by week 4 (Appendix K, G); however, many remained arrested as vegetative axillary buds with only prophylls visible (Appendix K, I). Primary AXMs of some IM plants developed into flower buds at node 2 and occasionally this flower bud became necrotic and failed to develop. By wk 4, the secondary vegetative axillary

buds on DUN plants had initiated a second leaf pair (Appendix K, H) whereas secondary vegetative axillary buds on IM plants had not and, in some cases, had not yet initiated prophylls (Appendix K, I, inset). Secondary axillary buds at node two from both populations were always vegetative, and never grew out into branches (Baker and Diggle, 2011).

## APPENDIX L



Appendix L. Results for *MAX1* expression in Dunes and Iron Mountain populations of *Mimulus guttatus* – (A) Shoots (node 1). (B) All roots. (C) Roots, top half. (D) Roots, bottom half. Solid bars are Dunes (DUN) plants and striped bars are Iron Mountain (IM) plants. Asterisks indicate significant differences between DUN and IM expression levels.

**MgMAX1 expression in Dunes and Iron Mountain plants** – As predicted, DUN plants express *MgMAX1* at significantly lower levels than IM plants; however, IM plants do express *MgMAX1* at significantly lower levels than DUN plants in the root at weeks two and three. The pattern of *MgMAX1* expression may reflect the natural variation in DUN and IM branching phenotypes, which occasionally overlap. However, similar to *AtMAX3* and *4*, in *A. thaliana* reciprocal grafting studies demonstrate that the *AtMAX1* mutant phenotype (increased branching), can only be achieved when *AtMAX1* is non-functional in both roots and shoots (Turnbull et al., 2002). If *MgMAX1* function is similar to its *AtMAX1* ortholog, significantly lower expression of *MgMAX1* in both roots and shoots of DUN plants may be necessary to influence *M. guttatus* branching. Although *MgMAX1* expression is occasionally significantly lower in IM plants, it is only significantly lower in roots, never shoots. *MgMAX1* expression is significantly lower in DUN plants in both roots and shoots, however, these differences never occur at the same developmental time. *MgMAX1* expression may not be involved in branching phenotypes of natural populations of *M. guttatus*.